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Natural Biomaterials for Enhanced Oligodendrocyte Differentiation and Spinal Cord Injury Repair

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Natural Biomaterials for Enhanced Oligodendrocyte Differentiation and Spinal Cord Injury Repair

by

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Natural Biomaterials for Enhanced Oligodendrocyte Differentiation and Spinal Cord Injury Repair

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Spinal cord injury is a devastating source of suffering in the spectrum of human pathophysiology; advancement for clinical therapy in this area has been stagnant in comparison to modern medical development. Current treatments are palliative, and functional recovery is minimal. During the first two weeks after injury, dense glial scar forms that is impenetrable by regenerating axons. Intervention is imperative to minimize scar formation and provide a supportive environment for axonal regeneration. Oligodendrocytes are critical to maintain the health of growing axons during development and after injury. Obtaining these cells through differentiation of neural progenitor cells (NPCs) is a viable option, but current clinical trials involving stem cells are plagued by poor cell survival and undirected differentiation. Research indicates that local extracellular matrix (ECM) is vital to progenitor differentiation and tissue regeneration. During development, spinal cord ECM is comprised of high concentrations of laminin and hyaluronic acid (HA), which provide essential cues to direct NPC migration and differentiation.

The purpose of this research is to create a biomaterial optimized to direct NPC differentiation to oligodendrocytes. Natural biomaterials were optimized from distinct combinations of collagen I, HA, and laminin I to model the native ECM signals found during oligodendrocyte maturation. Four material combinations (collagen, collagen-HA-laminin, collagen-HA, and collagen-laminin) were fabricated into injectable hydrogels to mimic the range of compressive and shear mechanical properties present in neonatal central nervous system (CNS) tissue. Differentiation was assessed by culturing rodent fetal NPCs in these materials without specific soluble factors to direct cellular behavior. The three-component hydrogel performed optimally and achieved a 66% oligodendrocyte differentiation rate compared to approximately 15% in the collagen alone hydrogel.

An *in vivo* study was then conducted using a rat contusion model of spinal cord injury with intervention using the injectable, three-component hydrogel seeded with rat NPCs. Functional recovery was assessed using six behavioral tests. Significant recovery was observed using two behavioral tests six weeks post-treatment. Lesion size was measured and correlated well with behavioral outcomes. The data obtained in this research indicate that a multi-component hydrogel mimicking native, developmental CNS tissue may address problems associated with current clinical practice.

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Chapter 1: Introduction and Background[1][2]

Spinal cord injury (SCI) is a devastating source of suffering in the spectrum of human pathophysiology. Notably, outcome improvement has remained stagnant in comparison to other clinical advancement throughout the history of modern medicine; currently treatments are palliative and functional recovery is minimal [1]. During the first 2 weeks after injury, a dense glial scar forms that is impenetrable by regenerating axons. Approaches to minimize scar formation and provide a supportive environment for axonal regeneration are imperative to improving outcomes after injury. Oligodendrocytes have been shown to be critical in maintaining the health of growing axons during development and regeneration and are important in retaining axonal function locally after injury [2, 3]. Obtaining these cells through differentiation of neural progenitor cells (NPCs) is feasible, but current clinical trials involving stem cell transplantation are plagued by poor cell survival and undirected differentiation in vivo [4]. Research indicates that local extracellular matrix (ECM) is critical to direct cell differentiation during development and healing. During development of the mammalian nervous system, the spinal cord ECM is comprised of high concentrations of laminin and hyaluronic acid (HA) [5, 6]. Laminin is a major component of the basement membrane of blood vessels in the mature tissue [7] and has been reported as an adhesive component associated with glial cell migration [8-11]. Conversely, HA is non-adhesive to mature cells but presents active

[1] Parts of this chapter follow closely from a prior publication, of which I am a co-author, [27]. Dr. Khaing and Dr. Thomas each contributed to sections of the paper as well. Dr. Schmidt acted as the mentor and directed us in the writing of this review article.

[2] Parts of this chapter follow closely from a prior publication, of which I am first author, [57]. Dr. Schmidt and Dr. Schallert acted as my mentors and directed me in writing this review article.

migratory cues to progenitor cells [5, 12, 13]. HA has been applied to spinal cord injury to reduce scar formation and inflammatory response [14, 15]. These components provide critical cues to neural progenitor cells during development to direct migration and differentiation.

Though researchers have attempted to treat spinal cord injury with NPC transplantation, few studies have produced robust results indicating successful differentiation of the transplanted cells and many transplanted cell populations exhibit low cell survival. Merging the fields of biomedical engineering and neuroscience to approach this problem may be the most successful option. The purpose of this research is to develop a biomaterial optimized to direct NPC differentiation to oligodendrocytes based on cues present during development.

STATISTICS OF CLINICAL SPINAL CORD INJURY

Over one million people suffer from paralysis due to spinal cord injury [4]. This is an injury most commonly sustained by healthy individuals between the ages of 19 and 29. Therefore, many patients endure decades with a severely decreased quality of life following their injury. The most common causes of spinal cord injury in the United States are work-related and motor vehicle accidents, however over 16% of these injuries are sustained during sporting or recreational outings [1, 4]. This injured population introduces novel issues in that they derive from an active and healthy subpopulation, rather than the elderly or other groups that are chronically prone to injury and require frequent medical intervention. Furthermore, most spinal cord injury patients have an opportunity to receive treatment at an age where the likelihood of healing and

regeneration is relatively high. These young and otherwise healthy patients are a promising population for various treatment strategies.

With many sufferers sustaining their injury at a young age, the lifetime cost of the spinal cord injury can be very high. In the first year, treatment costs can range from $300,000 to over $1,000,000, depending on the level of the injury. Each following year, costs range from $40,000 to over $150,000 [1, 4, 16]. These numbers suggest that the lifelong treatment for one patient can cost more than $3,000,000. Patients pay much of this price out of pocket. However, government and private health benefits also assume significant monetary burden for these patients. New treatments for spinal cord injury could increase patients' quality of life and reduce governmental healthcare spending by 400 billion dollars in future costs [1, 4, 16].

Spinal cord injury patients experience different levels of paralysis depending on their injury level. If the injury is contained below the first thoracic vertebra, the patient can experience a range of effects, including complete or partial paralysis of the legs and complete paralysis of abdominal muscles up to the nipple level, resulting in an inability to sit up straight without assistance. A person with an injury at or above the first thoracic vertebra will suffer quadriplegia or tetraplegia, meaning full or partial paralysis of all four limbs. In addition, patients with this level of injury can experience difficulty breathing, impaired coughing and clearing of the chest, as well as instability while sitting [4, 17].

Paralysis of the musculoskeletal system is the most readily observable pathology associated with spinal cord injury. However, decreases in sensitivity of the sensory nervous system also occur. Decreasing sensitivity increasing a patient's risk of disease or

injury by diminishing their responsiveness to potentially harmful. One example is "bed sores," or necrotic tissue at locations where body weight has been applied over a long period of time without sufficient movement or circulation [4, 17]. Prolonged pressure and resulting ischemia from immobility causes the skin and surrounding tissue to die and can lead to deep open wounds, increasing the risk of infection. Sensory and motor dysfunction can also affect an individual's mental health. These patients must learn to depend on assistance to survive and may find the experience humiliating and degrading.

People who have sustained spinal cord injuries can also suffer from bladder and bowel dysfunction. To manage these symptoms, people with a cervical or high thoracic injury often require catheters or attached urine bags and the execution of a daily bowel plan [4, 17-19]. If the injured person has a lower level injury, they are often able to perform many necessary tasks on their own after receiving instruction on how to perform the movements appropriately. In the first few weeks of learning, they must rely on assistance to tend to their bowel movements. Such invasive requirements forces these patients into a situation where they lack personal space and report feelings inherent to a compromised personal dignity [4, 17, 18]. Bladder and bowel issues are some of the least discussed side effects associated with spinal injuries, and people who have not directly dealt with paralysis often overlook these issues [4]. Additionally, many spinal cord injured people suffer sexual dysfunction in relation to sensation, as well as degradation or complete eradication of function in the sexual organs [4].

When the injury is at the 5^{th} or 6^{th} thoracic vertebra, the body experiences something called autonomic dysreflexia, also called hyperreflexia [4, 17, 20, 21]. This

condition can be life-threatening. This phenomenon results in the autonomic nervous system overreacting to issues that the spinal cord injured person cannot otherwise sense. For example, the body can respond to a full bladder by causing the person's blood pressure to rise excessively, leading to a faster heartbeat, tingling around the face and neck, blotchy skin, a pounding headache, and even fever. This reaction can be fatal in some cases if treatment is not sufficient [17]. Furthermore, this condition can occur in situations that would otherwise not be adverse, such as a full bladder or a sub-lethal bacterial infection. This reaction can also occur during sexual activity or other pleasurable behaviors [17].

When patients with spinal cord injury were surveyed to determine what features of their injury they would like treated, or which area of dysfunction they would like addressed first, they gave some unexpected answers. Quadriplegic patients requested that research focus on regaining bowel and bladder control, followed by sexual function. After these two choices, arm control trailed on the list as the next most important recovered ability, followed by a return to walking. Similarly, paraplegics also ranked bladder and bowel dysfunction above sexual dysfunction, followed by walking [4, 22].

SPINAL CORD CELL TYPES

To understand the downstream healing response after spinal cord injury, it is essential to first understand the native cell types of the central nervous system (CNS). The CNS has two categories of cell types: neurons and glia. Neurons are the signal-conducting cells that respond to chemical and electrical stimuli to transmit information throughout the brain and spinal cord and communicate to the peripheral nervous system.

It is critical that these cells be protected from infection and damage, and neurons are frequently the focus of repair strategies for CNS disease or damage [23-25].

Glial cells function to enhance the capabilities of the nervous system by interacting with the systemic environment and increasing the rate at which neurons can transmit signals. There are three types of glial cells: astrocytes, microglia, and oligodendrocytes. Astrocytes are heavily involved in the blood brain barrier and blood spinal cord barrier, with tight junctions formed between astrocytes and endothelial cells in blood vessels. Astrocytes shuttle nutrients and chemical signals from the circulating blood to neurons and CNS tissue [9, 10]. In normal physiological conditions, very few molecules can move from blood to the CNS tissue passively. This is an obstacle to delivering therapeutics to the CNS, but is important to maintain a thriving CNS environment. Additionally, this barrier prevents access by the systemic immune system. Endogenous CNS macrophages, termed microglia, are distributed throughout the CNS tissue and act to clean debris and damaged cells. These phagocytes are particularly important when cells of the CNS die, as they remove components in the extracellular fluid that can affect regenerative [9, 10, 26].

Astrocytes also function to absorb excess chemical signals around synapses, thus providing maintenance of the neurotransmission mechanisms [10, 27]. These functions are important to maintain the healthy environment of the CNS, as well as to enhance the neurotransmitter signal by preventing dilute signal from remaining in the synapse area [10, 27]. After injury to the CNS, astrocytes recreate the blood spinal cord barrier by creating a glial scar to prevent invading cells and chemicals from entering the undamaged

tissue. However, glial scar also creates a barrier to axonal growth necessary for restoring function [10].

The final glial cell type of the CNS are oligodendrocyte, which act as the myelinating cells to increase electrical conduction speed of signals along axons. By wrapping the axons in myelin, oligodendrocytes enhance signal conduction to enable nearly immediate responses to sensory input and fast motor movement initiation [25, 28]. Oligodendrocytes also provide myelin associated protein signals that prevent axonal growth. Although beneficial in normal physiology to prevent aberrant sprouting, these inhibitory proteins are released into the environment when oligodendrocytes die, preventing axonal growth [29]. Endogenous and invading macrophages can assist in clearing the inhibitory myelin signals, but signals frequently persist after injury, contributing to axonal dieback [10, 26]. Oligodendrocytes and oligodendrocyte progenitor cells can respond to these signals and uptake myelin-associated molecules to assists in clearing the injury area [29].

Oligodendrocytes mature during the neonatal period of development [30]. During this time, the extracellular matrix is comprised of high concentrations of hyaluronic acid (HA), laminin I, and collagen IV, among other components [7, 8, 31, 32]. These molecules have been associated with oligodendrocyte progenitor cell migration [31, 32]. Hyaluronic acid has been implicated as a glycosaminoglycan that prevents the maturation of oligodendrocytes to a myelinating phenotype [33], however, it is supportive of NPC migration and proliferation. Hyaluronic acid concentration decreases during neonatal development, this decrease is followed by oligodendrocyte maturation [5, 12]. The

transition of ECM component concentrations during development provides timed cues to cells to enhance differentiation and cellular interaction. Notably, this component of the extracellular matrix has a decreased concentration in mature tissue correlating with the maturation of the axonal myelination in the spinal cord [34, 35]. Such findings indicate the importance of extracellular matrix signals in directing differentiation and maturation during development.

SPINAL CORD INJURY

A significant part of this section follows closely with a previously published manuscript with the same author [27].

Cells respond to signals at all stages of development, the lack of pro-regenerative cues after SCI may contribute to the failure of spinal cord regeneration.

After spinal cord injury, the blood spinal cord barrier is damaged causing infiltration of immune cells, fibroblasts, blood, and other external factors and cells to the CNS tissue [26]. These invading cells can cause cell death, axonal dieback, and damage to healthy tissue near the lesion. The astrocytic scar assists in preventing this passage of invading signals into the CNS. To create a viable barrier, the astrocytic scar forms around a fluid filled cavity, sealing the healthy CNS tissue from the exogenous factors (see **Fig. 1.1**).

Primary axonal death occurs acutely after injury in response to the trauma. During cavity formation, axons retract, or dieback in response to exogenous and endogenous signals. This dieback increases the cavity size and decreases the possibility for regeneration [10, 26, 36].

Astrocytes and oligodendrocyte progenitor cells create a scar to prevent exogenous factors from entering healthy spinal cord tissue. Astrocytes within the scar are considered reactive and are identified by hypertrophy, process extension, and increased glial fibrillary acidic protein (GFAP), vimentin, and nestin expression [37]. Hypertrophic oligodendrocyte progenitor cells increase their production of NG2 and localize to the scar as well. If the membrane around the CNS tissue known as the dura is broken, invading meningeal cells, infiltrating macrophages, and activated endogenous microglia contribute to the scar [38, 39]. Schwann cells from the local dorsal roots have been observed invading the injured area as well [40]. The presence of Schwann cells is associated with tissue sparing and remyelination [41, 42] but they are indicative of increased trauma with dural breakage.

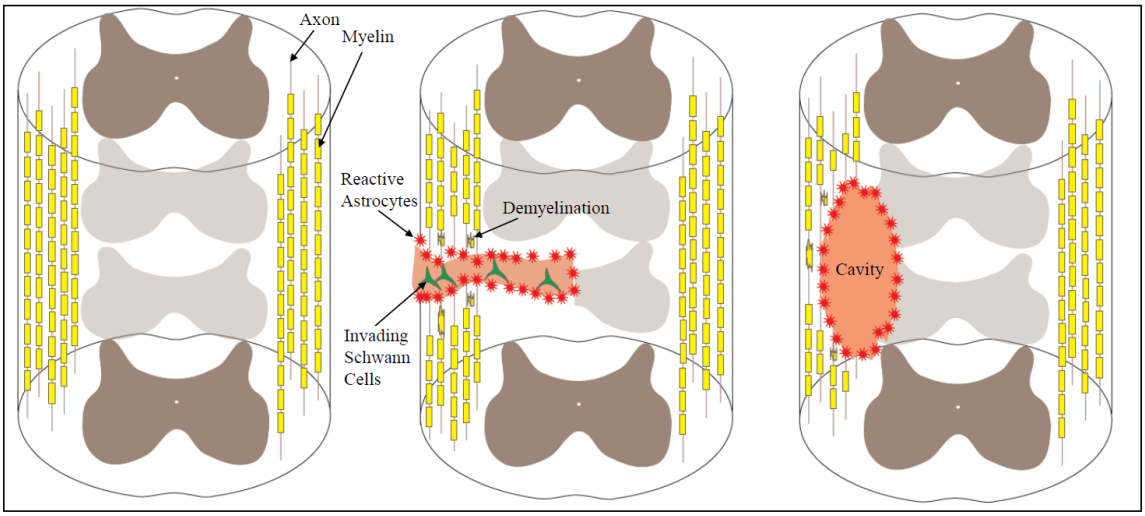

Figure 1.1: Spinal cord injury models.
In healthy spinal cord tissue, myelinated fibers run along the white matter surrounding the gray matter. After a transection injury, reactive astrocytes form a scar around the created cavity and Schwann cells and other exogenous cells infiltrate the area. After contusion injury, a fluid filled cavity forms surrounded by astrocytes.

The glial scar matures over the first two weeks after injury and is partially comprised of proteoglycans such as neurocan, phosphocan, versican, and brevican [40]. One notable component of glial scar tissue are chondroitin sulfate proteoglycans (CSPGs). CSPGs create an impenetrable barrier to axonal growth in many neuronal systems [43]. Moreover, myelin-related glycoproteins have been associated with the development of an inhospitable environment for the severed axons of the CNS [44-47]. These factors constitute a formidable environment for regenerating axons. Conversely, astrocytes activated at relatively long distances from the traumatic injury act in favor of regeneration [48]. These cells are characterized by an increase in size and transformation into a more pronounced stellate shape; they also produce soluble trophic factors that enhance the local survival of neurons and glial cells.

Although inhibiting formation of scar after injury appears to provide a solution, it is important to note that formation of scar tissue after injury to the CNS reestablishes homeostasis. Increases in inflammatory response, axonal dieback, and lesion volume were found in mice with targeted depletion of mitotic astrocytes [49]. This indicates the importance of a robust glial scar response. Additionally, components associated with re-establishing the blood brain barrier after injury have been shown to provide support in the chronic injury environment. These factors make inhibition of the formation of scar an incomplete option. The cells creating the scar respond to the environment around them, so providing signals to minimize their activation while also delivering growth supportive cues to the axons may be a more complete option.

Myelinating oligodendrocytes are damaged during the traumatic injury causing demyelination in the core of the injury (see **Fig. 1.1**). The axons directly affected by the trauma dieback, yet so do axons that have become demyelinated following injury. As mature oligodendrocytes die, endogenous oligodendrocyte progenitors are recruited to the area of injury, however, this population is limited in that there are too few progenitors to remyelinate the dying axons. In addition, dying oligodendrocytes release molecules into the extracellular space that normally prevent aberrant axonal sprouting in healthy tissue, therefore creating an excess of myelin-associated proteins (MAPs) that prevent regeneration. Macrophages, microglia, and oligodendrocyte progenitors are needed to remove these inhibitory cues from the injury environment [26, 50].

If the blood spinal cord barrier is broken, endogenous oligodendrocyte progenitor cells become hypertrophic, increase their expression of NG2, a CSPG receptor, and contribute to the scar. However, local oligodendrocytes also increase their production of molecules that encourage growth, leading researchers to believe that they may be vital in creating a growth permissive environment for regeneration [10, 51]. Remyelination has become an exciting topic for researchers in this area. Treatments that encourage remyelination and prevent demyelination decrease cavitation after injury and increase functional recovery [25, 52]. Cell replacement therapies [2, 52, 53], and pharmaceutical treatments [3, 54-56] have been used to attempt to increase myelination after injury, but providing effective remyelination has proven difficult. Limitations to the attempted techniques include low cell viability and undirected cell behavior as well as low local

retention of pharmaceuticals causing only short term effects. To study the effects of such treatments, researchers have turned to in vivo animal models.

RESEARCH MODELS OF SPINAL CORD INJURY

A significant part of this section follows closely with a previously published manuscript with the same author [57].

When determining which animal model and behavioral tests to use, researchers have many factors to consider, including the injury being modeled, species-specific differences, cost, and feasibility of analysis. The detail that can be attained using alternative behavioral tests should not outweigh the importance of standardized measurement between research groups with similar goals. Comparability of the models and assessment is critical to translate treatment to the clinic. Further, it is important to recognize the importance of planning. Researchers should choose models to meet their goals rather than performing a barrage of tests and reporting the most significant findings.

The therapeutic goals should match the damage expected by the model [58]. For example, if a treatment is designed to specifically connect rubrospinal tract neurons, a moderate midline contusion injury will not suffice to cause much damage to that region. The region of interest also needs to be chosen wisely to achieve specific deficits and to allow for recovery.

Another important aspect to consider is cost. Different injury models may be much more expensive to implement. The Infinite Horizon Impactor is much more expensive than a set of aneurysm clips, and a transection model can be performed with

standard surgical instruments, making it even less expensive. Furthermore, the cost of the species used, and the cost of post-operative animal care should be considered.

Behavioral assessment should be performed to assess functional deficit and recovery in combination with histological assessment. Behavioral assessment can elucidate the influence of the damage and repair of the tissue on overall function. Different models can make some analysis more easily performed or relevant; for example, transection models can be advantageous to eliminate axon sparing, but sprouting and axon sparing are more relevant in a contusion model. Transection models create a space for placement of pre-formed biomaterials, but if a treatment is injectable, a contusion model may be advantageous to more closely mimic clinical injuries.

It is important to consider the leading causes of morbidity and mortality among rodent test subjects. Autophagia is common for all injury models [59, 60]. Respiratory tract infections and decubitus ulcers are also concerns for researchers working with cervical SCI [61]. Urinary tract infections are less prevalent with cervical than with thoracic SCI models but should still be monitored [61].

Rodent models cannot predict exactly how human patients respond to SCI physiologically or functionally; however, they provide an early assessment platform for treatment and physiological damage after SCI. Human SCI is very complex and there is an appreciation among researchers that the models cannot mimic the injuries perfectly, nor can they expect all behavioral symptoms observed in humans to have correlates in animals, or vice versa. It is important to examine behavioral deficits in rodents, even without a human correlate, to determine if there is a functional change. Although humans

may not show an obvious vibrissae-elicited placing reaction, this test can reveal a loss, and a return, of sensory and motor communication between the brain and spinal cord. An animal's ability to perform in different tasks can reveal specific tracts that are regenerating, even if they are not in the same location in the human spinal cord. This can reveal a promising treatment that can then be tested further on higher species or directly on humans in a hope to help with recovery. It must be kept in mind that treatments that rescue neurons from degeneration may sometimes have an adverse effect on functional outcome [62].

Types of injury

A significant part of this section follows closely with a previously published manuscript with the same author [57].

Rodents are commonly used for spinal cord injury models to help predict functional outcomes of neurological disorders and injuries. Many of the functional behavior outcomes appear after SCI in rodent models parallel clinical symptoms observed in human SCI patients to a remarkable degree. About 62% of clinical spinal cord injuries are at the cervical level [16]. Therefore, there is a desperate need for unilateral cervical spinal cord injury rodent models to assess the potential of treatments to help the majority of SCI patients regain function. In addition, treatments aimed at improving functional recovery can be more adequately evaluated by using different injury models and sensitive behavioral assays that target the site and extent of injury. Some research models are considered more clinically relevant than others; however, it is important to remember that human SCI is very complex, and many different injury types

occur in human patients. Understanding the strengths and limitations of the animal models will allow more relevant analysis of the injury, behavioral sequelae, and therapeutic approaches.

There are three main surgical injuries used to model SCI: contusion, compression, and transection. In rat models, contusion and compression injuries form a cavity after injury as the glial scar forms and demyelination occurs (see **Fig. 1.1**). Transection surgical models introduce an immediate artificial cavity which quickly acquires similar glial scarring and infiltration of exogenous cell types (see **Fig. 1.1**).

Contusion and compression injuries are the most common forms of SCI in humans. Researchers can create these injuries using balloon compression, spring-loaded clips, and computer-controlled impact. These devices can be controlled for force of impact and dwell time that the force is applied. Each contusion model has strengths and weaknesses (see **Table 1.1**). This allows researchers to tailor the injury to their specific goals.

Balloon compression models are advantageous because they can precisely control the time and force of the compression and can be performed percutaneously, without a laminectomy. This model mimics a burst intervertebral disc or cystic injury in humans. The compression models used in rodent models are often not considered relevant because the decompression occurs much earlier than would occur in clinical SCI cases [63-65]. Spring-loaded clip models also allow good control of the time and force of the injury and are easy to perform with precise placement [66]. This is one of the most inexpensive options for compression or contusion injury models. However, clip compression models

require extensive decompression of the spinal canal, which may cause further damage or inflammation. Compression injuries can also be performed with modified surgical forceps to create a reproducible functional deficit, and are more commonly used in a thoracic injury model [67, 68]. Static weight compression is another model in which a known weight is placed gently on the spinal cord and held there for a controlled period of time [69]; because there is less acute damage, this model is not as clinically relevant, but still provides important information regarding the damage of compression alone. Taken together, the balloon and spring-loaded clip models are especially useful in assessing the most optimal decompression time after injury [63, 64, 70] and have helped change the treatment of human patients after SCI. Prior to 1997, surgery was not performed as quickly as possible on patients in fear of doing further harm, but currently, patients are rushed into surgery to remove pressure from the spinal cord to prevent further damage [64].

Although transection models are considered less clinically relevant, they allow researchers to precisely affect tracts and enable the most accurate assessment of functional recovery with the ability to tailor behavioral tests based on the axon tracts affected. Dorsal hemisection [71], dorsal quadrant transection [72], lateral hemisection [73], dorsal column transection [71] have been performed. These models are especially useful for assessing axon regeneration because the amount of axon sparing should be minimal or controllable, especially if a complete transection is performed. Transection models are usually performed with precision to remove specific tracts or areas of tissue. For example, if researchers wanted to compare and assess the ability of various treatment

options to support regeneration of the rubrospinal tract, a quadrant or lateral hemisection can be very useful to guarantee that no axon sparing occurs. SCI lateral hemisection models include a lesion that removes half of the tissue laterally and is generally performed manually by the experimenter. This model has similar deficits as the human Brown-Sequard syndrome, in which half of the body loses function, whereas the other half of the body retains function [74, 75]. Researchers have used transection models to examine multiple treatments, including biomaterials [76], drugs or growth factors [77], and stem cell transplantation [78]. Dislocation [79], and ischemia [80], have also been modeled anatomically and behaviorally.

Computer-controlled impactor injury models are generally recognized as the most clinically relevant contusion models [81] and are well suited for measuring functional deficit. There are many different devices available for this model: (1) the MASCIC device uses a weight drop technique with different heights to control the severity of the lesion and has been well characterized for thoracic SCI [58], (2) the OSU device controls the displacement of the head to control severity of the lesion [82, 83], (3) the PinPoint device from Hatteras allows control over velocity and displacement, and was originally described for thoracic injury [84], and (4) the Infinite Horizon Impactor, which controls applied force to control the severity of the lesion and has been thoroughly characterized in both thoracic [85] and cervical SCI [86, 87]. These approaches can all be used to create reproducible lesions; however, the necessary equipment can be expensive to purchase and the techniques require extensive training for proficiency. These models have been used to examine functional deficits and correlate them to histopathology [86, 87] to examine

demyelination [88], and as a means to assess biomaterial delivery [89] and cell delivery [90] for repair and regeneration.

Table 1.1: Pros and cons of surgical animal models.
Differences in surgical models allow researchers to choose the surgical model to best study their goals.

Injury Model	Pros	Cons	Clinical Correlates
Compression Balloon Spring-loaded clip Modified forceps Static weight	Control of time of application Control of force of application Useful to assess decompression time Inexpensive	Decompression occurs much earlier than in clinical settings Extensive lamina removal causing extensive decompression	Intervertebral disc burst injury Determine optimal decompression time
Computer-controlled impactor contusion MASCIC OSU Infinite Horizon PinPoint	Reproducible lesions Useful to assess demyelination and axon sparing Considered most clinically relevant model	Expensive	Considered most clinically relevant model
Transection Dorsal hemisection Dorsal quadrant Lateral hemisection Dorsal column	Useful to assess axonal regeneration Inexpensive	Considered least clinically relevant	Stab or shooting injury Brown-Sequard syndrome

Behavioral tests

A significant part of this section follows closely with a previously published manuscript with the same author [57].

Researchers also need to determine which behavioral tests are most relevant for their species, model, and study goals. Just as models will show deficits based on which tracts are affected, behavioral tests are sensitive to specific functional deficits. For example, if a model damages the corticospinal tract only, the animal's locomotion should not be seriously affected, so behavioral tests examining locomotion should not be exclusively used. Similarly, physical therapists and physicians examine human patients using the American Spinal Injury Association (ASIA) scale to look at specific deficits. This scale is used to determine the extent and level of injury for patients [17]. Patients are rated on specific functions that are associated with levels of the spinal cord (e.g., C5 is associated with bicep strength) and given a score of 0-5 where 0 is complete paralysis, and 5 is normal function. Scores of all of the tested functions are then combined to give them an overall score from A-E where A is complete paralysis (including sensory function) and E is normal function. Behavioral tests should be chosen to assess a range of functionality relevant to the injury model.

Vibrissae-elicited placing is a commonly used test to examine the sensorimotor function after spinal cord injury in rats [73, 91]. This test involves stimulating the whiskers on a table edge to elicit a motor response of the animal placing its forepaw onto the table to gain stability. A graded score of 0-4 is assigned based on the extent of movement of the limb being tested where 0 describes complete paralysis, and 4

represents normal function. This test can indicate the integrity of motor and sensory tracts. This test eliminates the possibility of a reflex response being elicited by stimulating a sensory route not directly associated with the forelimb. Therefore, this test can reveal that a motor tract is intact along the distance it travels from the brain. Based on the level of function, this test may also reveal the extent of damage to the cross-sectional area of the motor tract.

Contact placing is similar to vibrissae-elicited placing and involves a light touch to the paw from which the motion is being elicited; this test has been performed on rats [92] but is difficult to perform on mice. This involves a light touch that does not signal the pressure sensory nerves in the skin to elicit a response, and the experimenter must be careful to not move the limb or any joints during testing. This test can show if there is a reflex; however, it will not differentiate between a reflex and a complete tract connection, where the tactile information is transmitted to the brain prior to eliciting the response. This test is similar to the tests performed for the ASIA scale used for SCI patients to determine if they can feel light touch. In human patients, they are asked to tell the doctor if they feel the light touch; however, with animals, that is not possible, so this test incorporates motor movement as the signal to show that the light touch is perceived.

Proprioceptive placing indicates whether the reflex arc is still present. If the animals are able to respond to light touch, this test will not work because they will respond before there is motion of the limb. The test has been performed on rats [92] but is difficult to perform in mice. By stretching the tendon as a joint is extended, a reflex

response is elicited. This is similar to the reflex tests performed for the ASIA test in human SCI patients.

Limb use asymmetry during vertical-lateral exploration in a cylinder (20 cm diameter, 30 cm height for rats; 11 cm diameter, 20 cm height for mice) is an easy, inexpensive test to determine whether the animal has a preference in its limb use; this test has been used extensively in rodents [73, 87, 91, 93-95]. The cylinder test involves allowing the animals to move naturally and to vertically explore the walls around them while the experimenter records which limbs are used for weight supported stepping on the wall. This allows a clear determination of sidedness in natural function. This would allow experimenters to determine which side of the animal was more affected by the injury, and in the case of unilateral injuries, it allows the experimenters to determine the level of use of the ipsilesional limb versus the contralesional limb.

Swim tests can reveal functional deficits in forelimbs and hindlimbs. Normal rodent behavior when swimming to an escape platform consists of the rat holding both forelimbs forward under its chin in a planing position while stroking with its hindlimbs [96]. With a deficit in the forelimbs, an animal will not be able to plane their forepaws. This can result in one limb dragging. For cervical SCI injuries, this test is very reliable to determine presence of forepaw planing behavior, the angle of a limb that is not planing, and deficits in hindlimb stroking motions [93].

Open-field locomotion is used to assess the animal during normal functional movement. This involves observing and analyzing the position and weight support on individual forelimbs during uninterrupted locomotion. These tests are sensitive to the

amount of limb use when the animals are behaving naturally. One well standardized open field test is the Basso, Beattie, and Bresnahan (BBB) test [97]. Although, it is more appropriate for thoracic injuries, it has been used in injury models at a low cervical level as well [98]. There are a few open-field locomotion tests that are more specifically designed for cervical injuries, including the forelimb locomotor scale (FLS) [99], forelimb locomotor assessment scale (FLAS) [100], and another assay developed by a group at the University of Provence in Marseille, France [101]. Each test provides a score for different behaviors that are sequentially categorized based on forelimb function, including weight support on the limbs, paw position and orientation, and swinging motion. These tests observe animals in a similar situation to human locomotion because they do not use forced motion of the ipsilesional limb and allow the animal to compensate for the injury the way they usually compensate while moving on their own. Compensation occurs frequently in rodents and humans to increase speed, efficiency, or accuracy of a movement but does not exercise or demonstrate the functional ability of the ipsilesional limb.

Forepaw dexterity tests show fine motor movements and reveal paw strength and function. Animals can use their paws differently based on the model and the tracts affected. Similarly to human deficits, some animals cannot grip an object, and some cannot release their grip on an object. Some can move individual digits while others have only moderate wrist or shoulder function. A few pasta tests have been developed to reveal these differences. The Jones, Whishaw, and Bresnahan groups have developed tests to examine individual digit function [102-104], while the Schmidt group uses a

similar pasta type to examine overall forelimb function [73, 105]. Other handling tests use different foods, including macaroni pasta, peanuts, and grapes among others to examine functional deficits. Time to eat the object, forepaw use, and individual digit motion are all examined in these tests. Improvement can be quantified as the animals increase use of the ipsilesional limb. These are similar to human SCI patients' abilities to grip and manipulate objects. The ASIA exam includes finger strength and abduction assessment to determine injury level [17].

Forced motion tests have been developed to assess the functional abilities of each forelimb while preventing compensation with the other limb. The postural instability test (PIT) and a forelimb alternation test have been used to examine forelimb function during forced forward motion [73, 105]. In this assay, the experimenter holds the rat above a table with one of the rat's paws on the tabletop and moves the rat forward over its center of gravity. Then the position along this path at which the rat places its paw down after stepping forward is noted. Test scores depend on the distance the animal traverses before motion is elicited, while the animal is moved forward with only forelimb support, showing improvement as the score aligns with the pre-op distance. These tests allow researchers to examine an animal's ability to use the limbs while removing the chance for compensation with the other limb, giving the experimenter an understanding of actual functional abilities whether or not the animal has been compensating for that same motion. This is more comparable to clinical tests, as most of the tests performed on patients examine a patient's ability when they consciously attempt to make individual movements.

Grooming tests [86, 87] can be used for sensory-motor assessment. This test examines the rats' response to water applied on the face and upper back. The test is scored based on the animal's skill level and dexterity while grooming itself. Unlike the forelimb placing tests where the response is a reflex, the action during a grooming test is a conscious response to the sensory irritant. Grooming in normal animals follows a specific behavioral procedure, starting with licking the forepaws, cleaning the nose, face, behind the ears, and then the upper back. The rat's ability during this pattern of behavior determines the test score.

This is by no means a comprehensive review of rodent models and behavioral assays; many other functional behavioral tests exist. Additionally, this dissertation does not cover many sensory tests that should also be considered. The dot patch removal test [71, 91, 93], Von Frey hairs [60], and heat test [106] can all elicit responses to sensory input and may provide insight to hyperalgesia [107].

Many surgical models exist to study SCI in rodents; however, it is important to assess the goals and expectations of the experiment before choosing a model. Similarly, behavioral tests need to be chosen carefully to ensure that a researcher is examining a relevant function. It can be tempting to use a barrage of tests and report the tests that show the most promising or significant results, but this approach to determine the recovery after SCI is not scientifically sound. Each aspect of a study should be planned before the experiment, and a pilot study is recommended to provide insight into what is expected from each model and treatment to minimize animal use and unnecessary experimental time during the experiment.

CURRENT RESEARCH ON SPINAL CORD INJURY

Current research includes the examining the anatomical effects of spinal cord injury (SCI) and the cellular and extracellular matrix response to the injury. The reduction of scar formation is important to prevent and treat demyelination, and to encourage regeneration. The scar that forms after spinal cord injury segregates that injury area from the spinal cord, preventing toxins from entering the healthy spinal cord tissue, but also prevents regenerative growth of axons. Minimizing this barrier to regeneration is critical to restoring function. Other research has examined mediating demyelination around the injury area to prevent axonal dieback and to encourage functional recovery after injury. Many different approaches have been taken to address regeneration and functional recovery after SCI; this dissertation discusses two treatment options, (1) cell delivery to and near the injury area and (2) biomaterial delivery.

Cell delivery

Several cell types have been examined to assess their potential to reduce scar formation, enhance tissue sparing and remyelination, and encourage regeneration including but not limited to Schwann cells [41, 108], olfactory ensheathing cells [109, 110], and neural progenitor cells [52, 111].

Schwann cells have been utilized for transplantation therapy after spinal cord injury in animal models since 1981 [112]. Many studies have examined remyelination and axonal regeneration with Schwann cell transplantation because Schwann cells are the myelinating cells of the peripheral nervous system. More recently, invasion of host Schwann cells after cell transplantation has also been considered as a contributor to

regeneration. The host Schwann cells behave similarly to transplanted cells providing natural signals to host CNS tissue to encourage regeneration [42]. Schwann cells used for transplantation are isolated from many sources, including adult and postnatal nerve and derived from induced pluripotent stem cells making them an exciting option for clinical translation. However, Schwann cell myelination exhibits a different phenotype than central nervous system myelin with inclusion of the Schwann cell cytoplasm. Each myelinating cell type expressed different proteins and morphologies during axonal interaction [113].

Biernaskie et al examined the effects of skin-derived Schwann cells on scarring after thoracic contusion injury in rats [108]. Cells were injected directly to the lesion epicenter to provide signals directly to at the scar site. There were increased numbers of axons growing through the lesion and myelination was more prevalent in animals treated with skin derived Schwann cells. Additionally, endogenous Schwann cells were recruited in high numbers to the lesion area to encourage regeneration. This study examined behavioral recovery after treatment and found an increase in Beattie Basso and Bresnahan (BBB) score with skin derived Schwann cell treatment, however, no increase in other behavioral test scores. This research showed an increase in spared white matter with skin derived Schwann cell transplantation. These results imply that the Schwann cells provided support to the myelinated tracts of the injured cord and possibly decreased demyelination, however, myelination of the growing axons was of the peripheral nervous system type [108].

After contusion cervical spinal cord injury in rats, Schaal et al transplanted Schwann cells to the injury area one week post injury [114]. They observed tissue sparing, more reticulospinal axons traced by anterograde dye injection to the lesion site implies growth and sparing into the lesion area. Additionally, in this study they assessed functional recovery. Recovery based on the BBB test was not observed, however, with forelimb grip strength, an improvement over untreated groups was observed. This is promising for Schwann cell treatment. This was an 8 week experiment to examine functional recovery and tissue sparing. Little growth was observed past the lesion [114], this may be related to the ECM deposited by Schwann cells, which has a high laminin content [115, 116].

The Bunge group out of the Center to Cure Paralysis in Miami, FL has extensively studied Schwann cell transplantation into rat models of SCI and is currently in collaboration with physicians performing clinical trials with this cell type in SCI patients. Pearse et al transplanted Schwann cells to the injured area of a contusion thoracic spinal cord injury model in rats and found dense regions of myelinated fibers where the transplanted Schwann cells were present [42]. However, the low cell survival (<5%) by the third week after transplantation, possibly related to the lack of ECM components in the lesion center, implies that the procedure requires further optimization. This research also showed evidence of invading Schwann cells in the contusion model used [42]. Invading Schwann cells have been shown to contribute to myelination in the lesion area after SCI [117, 118].

Schwann cells have also been applied to thoracic dorsal column transection where experimental groups treated with Schwann cells had increased numbers of myelinated axons traversing the lesion area. Imaizumi et al examined the effects of olfactory ensheathing cell and Schwann cell transplantation rostral and caudal to the injury area after spinal cord injury on electrophysiological function in rats [119]. Amplitude and number of action potentials detected increased with transplantation of each cell type indicating an increase in signal transduction. Assessing functional recovery in this way allows quantitative anatomical assessment of the therapeutic effects. The Schwann cell transplant group exhibited increased myelin fiber number and more thickly myelinated axons with peripheral phenotype, as was expected with the transplant type. Olfactory ensheathing cells are often studied as an example of a more central nervous system-type myelinating cell, however, their contribution to myelination is often limited. Transplantation of olfactory ensheathing cells results in an increase in axon number through the lesion area and tighter myelination compared to Schwann cell transplantation. In both cases, experimental groups had increased axonal growth and myelination compared to a lesion alone [119], implying that both cell types hold promise to restore myelination and protect tissue surrounding a lesion if transplanted after injury.

Olfactory ensheathing cell transplantation has been combined with behavioral training to examine functional effects [120]. The myelinating phenotype of the olfactory ensheathing cells provided support to the tissue surrounding the lesion and increased sparing of white matter while providing minimal remyelination. Though the animals were trained for 4-7 months after injury, there was little regeneration beyond the lesion site.

The researchers did find, however, increased white matter sparing and regenerated noradrenergic and serotonergic axons through the injury area. Functionally, the animals were scored using the BBB scale and found to increase their plantar stepping ability with olfactory ensheathing cell transplantation compared to media transplantation and training alone. A combined treatment of olfactory ensheathing cell transplantation and functional training further enhanced these effects, implying an importance for task-specific training [121].

Neural progenitor cell transplantation has also been recently explored as a treatment for spinal cord injury and may provide a more promising contribution to native myelin if differentiated appropriately. The cell populations used in these studies can be isolated from a variety of locations, including the forebrain to the spinal cord. Additionally, passages and culture conditions prior to transplantation are inconsistent between groups. Differentiation of these cells post transplantation has varying reported results, some researchers report high astrocyte populations [122, 123] while others report high oligodendrocytes [52, 124]. Many of the studies reporting high oligodendrocyte differentiation also include delivery of growth factors and differentiation directing agents to the injured area of the spinal cord. One of the most crucial issues documented in many of these experiments is low cell viability after implantation. Survival rate as low as a 4.6% have been reported [52]. However, the transplantation of these cells tends to provide positive results behaviorally and histologically, implying that even a small population of neural progenitor cells can provide a regenerative effect.

Parr et al transplanted adult neural progenitor cells to the lesion site of a thoracic compression injury model in rats nine days post injury and reported a significant increase in BBB score with neural progenitor cell transplantation [52]. A high percentage of the transplanted cells differentiated to oligodendrocytes (~63%), however, only about 4.6% of the cells survived for 7 days and the 12 week group showed an average of 1.2% cell survival [52]. Conversely, Vroemen et al reported neural progenitor cell survival after transplantation to the transected dorsal corticospinal tract, however, they did not report quantified results [125]. They reported significantly higher astrocytic differentiation of neural progenitor cells in this model. For this experiment, they examined cell proliferation after transplantation and reported minimal proliferation at three weeks post injury. Additionally, this experiment revealed the tendency of transplanted cells to remain around the edges of the lesion, most likely due to the lack of a supportive scaffold being present in the injury area [125].

Undirected differentiation is a significant problem with transplantation of neural progenitor cells. Pfeifer et al reported about 90% differentiation to astrocytic phenotypes four weeks after transplantation of autologous and allogenic adult neural progenitor cells to a cervical dorsal column transection model [126]. With neural progenitor cell transplantation, there was an increase in axonal sprouting to the lesion area compared to lesions with fibroblast transplantation [126]. This undirected differentiation may be controllable with transplantation of ECM signals with the cells. Signals similar to those present during development may lead NPCs toward specific phenotypes.

The Shoichet group recently applied neural progenitor cells rostrally and caudally to the lesion site of a thoracic compression model of spinal cord injury in rats [111]. They report a significant decrease in cavitation but low transplanted cell survival (0.4-1.2%). Many of these cells differentiated toward oligodendrocytes (~59%) when transplanted with an inert biomaterial functionalized with platelet-derived growth factor, indicating the importance of directing the differentiation of the transplanted cells toward oligodendrocytes. This experiment showed a 33% increase in axonal sparing when the neural progenitor cells were directed to differentiate toward oligodendrocytes compared to cells transplanted without specific signals [111]. This study introduces the possibility of transplantation with specific signals from a biomaterial, however, previously, researchers have transplanted cells with biomaterials that released growth factors. In most previous cases, the growth factors were not conjugated to the biomaterial. Lu et al transplanted neural stem cells in a fibrin matrix to a transection model of spinal cord injury and reported connectivity of the graft, axonal regeneration through the graft, and functional regeneration seven weeks after transplantation [89]. There was significant cell survival after transplantation and many of the neural stem cells differentiated along a neuronal lineage. A cocktail of growth factors were also included in the transplantation therapy, so the cause of this directed differentiation and cell survival is unclear [89]. The signals present directed cell behavior and provided a scaffold to prevent cell death.

These experiments provide significant motivation to examine cell transplantation in combination with biomaterials. The exciting results from experiments where NPCs

were transplanted with biomaterials also provide insight to methods that may assist in the direction of neural progenitor cell differentiation in vivo.

Biomaterial delivery

More recently, research has begun to focus on the mode of cell delivery in an effort to improve problems with cell viability [52], uncontrolled cell differentiation, and activation of transplanted and endogenous cells [120] after CNS injury. Biomaterial scaffolds can mitigate a number of these issues by eliciting desired cellular response by providing cues to cells during migration [127], differentiation [128], and regeneration [129, 130]. Hydrogels can provide benefit to the endogenous cells surrounding the implant site as well as the transplanted cells while acting as a delivery vehicle for cells into injured areas of the nervous system [89, 131].

Biomaterials for use in the CNS should create a permissive environment for growth, act as a suitable carrier for cells, and integrate into functional new tissue. Such biomaterials are usually applied in anatomically contained locations, such as in a SCI cavity [132] and used to deliver cells as replacement therapy to integrate into host tissue.

Many researchers report increased cell viability upon delivery of cells within a hydrogel along with other positive effects in many injury models. In a study discussed above using a complete transection model of thoracic SCI, Lu et al. showed success at increasing transplanted neural stem cell viability as well as functional recovery after spinal cord injury with the delivery of neural stem cells in a fibrin matrix compared to cells alone [89]. Patel et al reported increased cell viability when transplanted using an injectable collagen laminin hydrogel to deliver Schwann cells to the lesion site after

contusion spinal cord injury. This study systematically examined cell survival, proliferation, graft vascularization, and axonal ingrowth with and without the hydrogel delivery vehicle. By suspending the cells in a matrix prior to transplantation it was possible to observe increased cell survival (27% versus 14%) and an increase in axonal ingrowth and vascularization in the lesion area. These results indicate the importance of a supportive growth environment for regeneration. Using biomaterials as a delivery vehicle increased cell viability to encourage tissue replacement and regeneration [133].

Beyond effects on cell viability, it is important to examine how components and characteristics of the biomaterial used will affect cellular behavior. For example, when researchers implant stem or progenitor cells, it is advantageous to direct the differentiation of the cells toward a desired phenotype. In 2006, Engler et al examined cellular differentiation in response to matrix stiffness and found the differentiation of mesenchymal stem cells could be modulated by changing only the mechanical stiffness of the substrate on which they were cultured [128]. Differentiation toward bone, muscle, and neural lineage occurred on stiff (25-40 kPa), medium (8-17 kPa), and soft (0.1-1 kPa) substrates, respectively [128]. Following that work, other researchers examined the more precise differentiation of cell types within specific tissue types. Neural progenitor cells can differentiate along a glial lineage (astrocytes or oligodendrocytes) or along a neuronal lineage. Researchers have begun to delve into the specific mechanical environments most ideal for differentiation into each cell type. Seidlits et al showed that the mechanical properties of the material can direct differentiation of NPCs toward different neural lineages with hyaluronic acid-based materials with similar mechanical properties to brain

and spinal cord tissue [134]. Softer materials (~3 kPa) tended to direct differentiation toward neuronal lineage whereas astrocytes were the most prevalent cell type in materials with a higher compressive modulus (~5 kPa) [134]. Many of the experiments to assess NPC response to the mechanical properties of the environment were performed in vitro and not translated to in vivo models. The results provide insight to the response of stem and progenitor cells to differences in mechanical stiffness of the scaffolds without any other specific cues.

Oligodendrocyte response to mechanical cues has also been studied to determine the mechanobiology of demyelinating diseases [28]. Oligodendrocyte progenitor cells were examined in culture on substrates of varying stiffness to assess the effects of the mechanical environment on proliferation, migration, differentiation and survival. Cell stiffness, migration, survival, adhesion, and differentiation were assessed on polyacrylamide gels ranging from 0.1-70 kPa in compressive modulus. Though there was little correlation between cell stiffness and substrate mechanical properties, there was an increase in cell stiffness with differentiation was observed. In this study, researchers examined cell migration using time-lapse imaging and found that cells migrate more quickly on stiff substrates (70 kPa), proliferate most efficiently on 0.7-70 kPa substrates, and have the highest survival rates on intermediate stiffness substrates (0.4-1 kPa). To examine correlation of cell differentiation and substrate stiffness, this study used immunocytochemical markers for specific ranges of oligodendrocyte lineage and found that the most efficient substrate stiffness to develop mature, myelinating oligodendrocytes was 0.4-1 kPa [28]. This study elucidates the importance of the

mechanical environment on determining the behavior of oligodendrocyte progenitor cells and implies the mechanosensitivity of oligodendrocytes throughout development.

Aside from mechanosensitive properties of cells, cellular response to the chemical environment has also been extensively studied, particularly with regard to myelin development. Mothe et al, discusses the importance of chemical cues from the environment to direct differentiation and increase cell survival after transplantation and in culture [111]. In this study, growth factor cues were provided locally by conjugating platelet-derived growth factor to a hydrogel with a base of methyl cellulose, an inert, biodegradable compound. The hydrogel was optimized to increase oligodendrocyte differentiation, mainly by adjusting conjugated platelet-derived growth factor concentration [111]. They previously measured the hydrogel's response to shear stress and reported the storage modulus as less than 200 Pa [135], implying that these were soft materials that behave more like a solid than a liquid but do not provide significant mechanical stability. Additionally, the hydrogel composition included hyaluronic acid, a native extracellular matrix component discussed in more detail below, which provides cues to neural progenitor cells. The interaction of the immature neural progenitor cells with the hyaluronic acid may provide direction to the differentiation after transplantation.

Biomaterials are also delivered to impart effects on the surrounding tissue themselves. Hyaluronic acid hydrogels have been shown to decrease scar formation after transplantation into the spinal cord injury area [15]. In this study, a photocrosslinked hyaluronic acid hydrogel was transplanted to the injury site of a thoracic transection spinal cord injury. Astrocyte activation is limited after transplantation with hyaluronic

acid based hydrogels. Additionally, chondroitin sulfate proteoglycan staining showed a decrease in this molecule, which creates a chemical barrier to axonal growth. Approximately 50% fewer macrophages (ED1+) cells were present in treated groups compared to control injury groups on 1, 3, and 10 days [15]. This reveals some advantages to using specific molecules while designing a biomaterial for central nervous system regeneration, however, there are also disadvantages to using hyaluronic acid alone.

Hyaluronic acid is not innately cell adhesive. Immature cells migrate on hyaluronic acid, whereas adult cells do not express surface receptors capable of binding hyaluronic acid. Therefore axons and mature cells would not migrate in the area of a biomaterial made primarily of hyaluronic acid. Additionally, hyaluronic acid has been implicated in the inhibition of oligodendrocyte maturation [33, 34], so particularly with the goal of preventing demyelination and assisting with remyelination, the presence of hyaluronic acid should be controlled. Hyaluronic acid presence during the first two weeks of scar formation may be important to prevent proliferation and expansion of the astrocytic scar. Long term retention of HA can pose a detriment by preventing axonal regeneration through the lesion area, therefore diffusion of this component is optimal. Particularly with respect to oligodendrocyte differentiation, it would be advantageous if the hyaluronic acid could diffuse from the lesion area. Researchers have shown the importance of this particular extracellular matrix component with respect to neural progenitor cell migration [13, 35, 136]. These effects on cellular behavior parallel developmental levels of hyaluronic acid. During development, this component is present

in high concentrations in spinal cord extracellular matrix, whereas healthy mature spinal cord tissue has low levels of hyaluronic acid [13, 137].

Other native extracellular matrix components are important to direct cell behavior. Specifically, cells require adhesive components to migrate and regenerate during healing. During development, neural progenitor cells have been observed to migrate along forming blood vessels. These immature blood vessels have high levels of laminin I and collagen IV, which make up the basement membrane of the mature vessels [6-8, 138]. Such migratory cues during development may provide cues for differentiation as well as for axonal growth. One powerful example of the importance of such bioactive cues is the response of cells to the presence of laminin. Kofron et al examined the effects of laminin presence on neurite extension with CSPG presence and found the neurites extended near the CSPG coated substrates when laminin was present but not without that presence [139, 140]. Similarly, Yu et al examined the effects of laminin and chondroitinase, an enzyme that can erode CSPGs, on the bioactivity of CSPGs. Dorsal root ganglia neurites extend into the hydrogel that otherwise presented only growth inhibitory signals if laminin or chondroitinase was present [141]. These experiments indicate the importance of extracellular matrix cues on regenerative growth.

Biomaterials made of natural components that parallel the developmental extracellular matrix cues may provide an ideal environment to direct the behavior of cells when transplanted to the injury area after spinal cord injury. Although growth factors and chemicals can be used to direct cellular behavior in culture, they are impractical for sustained delivery to the transplant site. Delivery with biomaterials that provide natural

extracellular matrix cues may be an ideal way to stimulate cellular behavior locally in a sustained manner. By controlling the mechanical properties and composition of such biomaterials, differentiation can be directed in vivo after transplantation.

Considerations for transplantation

Delivery of a therapy to the injury site after trauma to the spinal cord needs to be considered. Pre-formed biomaterials can be placed in experimental transection models where the lesion shape and size is somewhat controllable, however, in contusion and compression models, as well as in the clinic, this is not a viable option. To deliver a preformed matrix to such models, secondary injury would be required as the surgeon would have to excise tissue above the lesion site to place the material into the cavity. Additionally, these preformed materials would not form to the injury site exactly, therefore, the edges would not all interact with the material. An injectable material would conform to the irregular shape of the injury, whether transection, contusion, or compression and provide an interface between the therapy and the scar tissue in the lesion [142].

Another advantage to injectable substrates is the ability to evenly distribute the desired therapy to the injury area and control the release. Injectable cells can be evenly suspended in the matrix prior to injection, providing a more favorable environment for cell interaction. With this interaction in mind, the matrix should also be designed to allow for cell infiltration after transplantation. Injection time is a factor that needs to be considered, fast gelling is optimal for in vitro experiments, but for in vivo transplantation a slow injection rate is necessary to prevent sudden pressure increase to the lesion area.

SUMMARY

There are many promising research areas for treatment after spinal cord injury, however to date, a viable treatment has not been developed. Many factors are important for the delivery of a therapy, but a combination therapy is needed to see significant results. Factors to consider when developing a combinatorial treatment include the interaction between the treatments and the timescales of interaction. Research has recently shifted from studying specific treatments to combining treatments and therapy delivery methods. With this development, the field of engineering has the technology to assist and collaborate with the neuroscience field to deliver cells and growth factors to injury areas after spinal cord injury. The use of biomaterials provides researchers with the ability to modulate more parameters and create a more favorable environment. Mechanical cues, chemical cues, cellular interaction, ingrowth, and distribution can now be controlled to deliver appropriate cues to the regenerating nervous system.

SPECIFIC AIM 1: OPTIMIZE A NOVEL INJECTABLE HYDROGEL COMPRISED OF EXTRACELLULAR MATRIX COMPONENTS TO MIMIC MECHANICAL AND BIOCHEMICAL PROPERTIES OF NATIVE SPINAL CORD TISSUE.

Develop protocols for multi-component hydrogel production with desired gelation kinetics for injectable in vivo delivery.

Tune mechanical properties of hydrogels to closely mimic native postnatal spinal cord tissue.

Examine hyaluronic acid release from the hydrogels.

SPECIFIC AIM 2: EXAMINE THE DIFFERENTIATION OF NEURAL PROGENITOR CELLS TOWARD OLIGODENDROCYTES EMBEDDED IN DIFFERENT HYDROGEL COMPOSITIONS.

Examine the effects of hydrogel composition on cell differentiation.

Modulate hydrogel properties to induce >50% NPC differentiation to oligodendrocytes.

SPECIFIC AIM 3: IMPLEMENT AN INJECTABLE HYDROGEL-CELL SYSTEM INTO AN IN VIVO MODEL TO ASSESS FUNCTIONAL AND ANATOMICAL RECOVERY AFTER SCI.

Develop a unilateral, cervical contusion spinal cord injury model to mimic clinical injuries.

Assess functional recovery after hydrogel and cell implantation using behavioral assays.

Analyze cellular and anatomical differences between groups after hydrogel and cell transplantation to find correlates between functional and anatomical recovery.

RATIONALE

Cells respond to chemical and mechanical cues to differentiate, migrate, and proliferate. Using biomaterials, chemical and mechanical properties of native tissue can often be mimicked to achieve similar responses. Progenitor cells have been transplanted into the injured spinal cord and have been shown to improve functional recovery [2, 52]; however, cell viability is low (~1.6%), and there is little growth of axons into the injured

area [52]. With a 3D matrix to deliver cells in vivo, cell viability can be increased and a growth supportive environment provided for axonal regeneration. Previously, the Tuszynski group has introduced human neural stem cells in a fibrin matrix to injured rat spinal cords and found that the cells promoted functional recovery [89]. Combining matrix components that are more native to the spinal cord (HA and laminin) should provide a matrix that mimics the native tissue more closely, thus directing differentiation along specific neural lineages.

Results from Specific Aim 1 provide a thorough understanding of the matrix mechanical property range that can be achieved using collagen-based composite hydrogels of this type. This allows a comparison between the hydrogel properties and those of native spinal cord tissue. Additionally, the examination of gelation kinetics and component release reveals a clear image of the properties of the hydrogels in vitro.

Results from Specific Aim 2 provide information to more clearly understand the cellular response to these hydrogels. These results elucidated which hydrogel composition can promote oligodendrocyte differentiation most effectively in vitro and revealed which hydrogel composition was most promising to translate to in vivo experimentation. A balance between mechanical properties and composition can direct differentiation toward oligodendrocytes.

Results from Aim 3 reveal the functional effects of these hydrogels on regeneration after spinal cord injury. By examining the functional deficits and recovery, the effectiveness of promoting regeneration can be determined, providing feedback to sensory and motor functional recovery ability. Implementing the hydrogel best

mimicking native spinal cord tissue and promoting oligodendrocyte differentiation provides feedback on the translatability of the engineered transplant graft.

Chapter 2: Biomaterial Development

SPECIFIC AIM 1: OPTIMIZE A NOVEL INJECTABLE HYDROGEL COMPRISED OF EXTRACELLULAR MATRIX COMPONENTS TO MIMIC MECHANICAL AND BIOCHEMICAL PROPERTIES OF NATIVE SPINAL CORD TISSUE.

The hypothesis of this work is that a hydrogel that mechanically and chemically mimics native neonatal spinal cord tissue should direct the differentiation of spinal progenitor cells toward oligodendrocytes

Rationale for hydrogel composition

The hydrogel composition was optimized from a collagen-based scaffold modulated with the addition of high molecular weight hyaluronic acid (HA) and laminin I. These components were specifically selected to affect the differentiation of neural progenitor cells (NPCs) to oligodendrocytes. Unlike more mature cell types, NPCs and oligodendrocyte progenitor cells express the surface integrin CD44 which binds HA, an otherwise non-cell adhesive component [12]. However, HA also prevents the maturation of oligodendrocytes [30] and was therefore not crosslinked into the hydrogel to prevent long term retention of this component in vitro and in vivo. Laminin is a cell adhesive component that is part of the basement membrane of the blood vessels in the developing CNS [6, 7, 143]. Developing NPCs migrate and mature in response to specific signals from these proteins, as evidenced by observations of oligodendrocytes maturation during the neonatal period of development [30, 144]. Neonatal tissue is soft (<1kPa) [28, 145] and has a high concentration of laminin I [6, 11, 138, 146] and high molecular weight HA [5, 13, 137, 147], signifying that these properties may be crucial to oligodendrocyte differentiation.

Collagen-based scaffolds were initially characterized by examining a range of collagen concentrations (1 mg/mL – 3 mg/mL) to assess compressive modulus. 1 mg/mL collagen hydrogels were extremely difficult to manipulate, and collagen hydrogels at higher concentrations (>2 mg/mL) had compressive moduli much higher than desired. Thus, 1.5 mg/mL collagen content was chosen to create hydrogels of similar compressive modulus to neural tissue. These findings align with similar results from previous studies [148]. Between-batch variability was assessed (n=6, repeated 3 times) to determine repeatability of hydrogel synthesis using a two-tailed F-test at a 90% confidence level. The batches did not differ significantly, therefore data from multiple batches can be confidently combined.

Hydrogel synthesis

Hydrogels were synthesized from rat tail collagen I (Corning, Corning, NY, CB354249), mouse laminin I (Trevigen, Gaithersburg, MD, 3446-005-01), and high molecular weight (1500 kDa) bacterial-synthesized hyaluronic acid (Sigma-Aldrich, St. Louis, MO, 53747). All hydrogels contained collagen to provide mechanical strength and stability. The collagen was diluted from the concentration provided by the manufacturer (10.08 mg/mL) to 3.75 mg/mL with 0.2% acetic acid, and collagen stock solution was then made by combining 4:1 (A:B), (A) 3.75 mg/mL collagen solution and (B) 5X DMEM in HEPES buffer. This solution was then vortexed for 10 seconds and stored on ice until use. Laminin was thawed slowly on ice or at 4°C and kept at the concentration of the stock solution provided by the manufacturer (6 mg/mL). Hyaluronic acid was dissolved at 15 mg/mL in deionized water overnight and stored at 4°C until use.

Hydrogels were fabricated by combining of the components at the appropriate concentrations (**Table 2.1**) with 1X PBS as a diluent to make four different hydrogel compositions: collagen (col), collagen HA laminin (Col HA Lam) collagen HA (Col HA), and collagen laminin (Col Lam). Final solutions were vortexed for 10 seconds and then centrifuged for 3 seconds to eliminate trapped air. All hydrogel synthesis work was performed on ice to prevent premature gelation. For cell culture experiments, 30 μL hydrogels were used and for mechanical characterization experiments, 120 μL hydrogels were molded in 8 mm diameter silicon molds (Grace Biolabs, Bend, OR). Hydrogels were placed in molds on parafilm for 40 minutes in a 37°C, 5% CO_2 incubator for gelation. When hydrogels were removed from the incubator, they were immediately placed in medium warmed to 37°C.

Table 2.1: Concentration of components in each hydrogel

Hydrogel Type	Collagen Concentration (mg/mL)	HA Concentration (mg/mL)	Laminin Concentration (mg/mL)
Collagen	1.5	-	-
Collagen HA Laminin	1.5	1.5	1.5
Collagen HA	1.5	1.5	-
Collagen Laminin	1.5		1.5

Methodology for mechanical characterization of hydrogels

Mechanical characterization was performed on hydrogels prepared as described above; formed in 8 mm diameter by 2 mm deep silicone molds and allowed to equilibrate at room temperature in PBS for one hour prior to testing.

Compressive modulus

Bulk compressive moduli of hydrogels were determined using an Instron apparatus (Model 3345, Instron, Norwood, MA). Cylindrical samples were compressed at a rate of 0.1 mm/s to at least 60% strain. Moduli were calculated as the slope of the stress versus strain curve in the linear region within the first 20% of the strain [134] (see representative data, **Fig. 2.1**). The total sample size per group was n = 15, achieved by performing three batches with n=5 per batch. Hydrogels were excluded from the study if they were torn or damaged during or prior to transfer to the Instron. Data from this process were compiled to determine the average compressive modulus of each hydrogel composition.

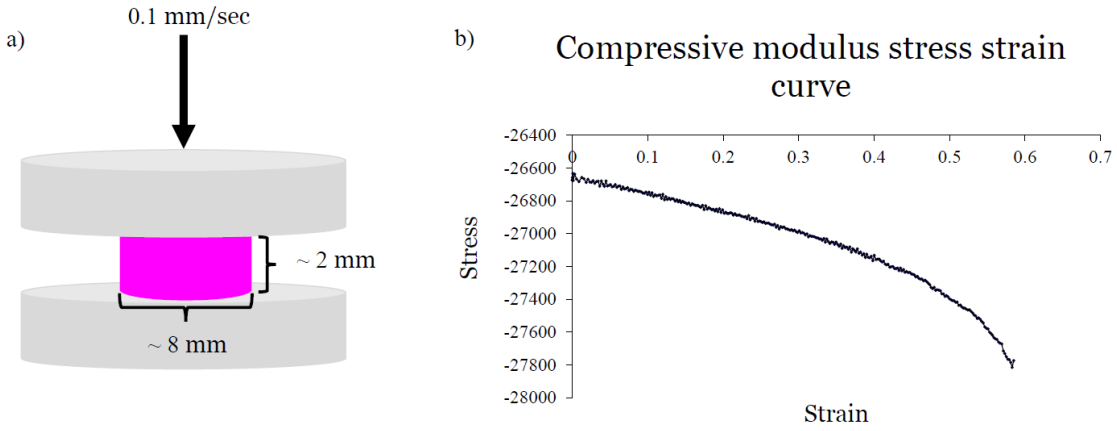

Figure 2.1 Compressive modulus.
(a) To assess the hydrogel response to compressive forces, hydrogels of ~8 mm diameter and ~2 mm height was exposed to compressive forces using an Instron. (b) Stress and strain were measured and plotted. From this, a linear region within the first 20% of compression was assessed to determine the slope of the line, which describes the compressive modulus.

Shear modulus

Hydrogel response to shear stress was determined using an Anton Paar MCR302 rheometer (Anton Paar, Graz, Austria). First, the hydrogels were subjected to an amplitude sweep from .01-100% strain at 6 radians/second to determine the linear viscoelastic range of the material (see representative data, **Fig. 2.2**). The median amplitude in that linear range, 5%, was chosen for subsequent frequency sweeps. Frequency sweeps from 0.1-100 radians/second were performed on all hydrogel compositions to determine the storage modulus of the hydrogel, which describes the elastic response of the material to shear stress. The total group size for each hydrogel composition was n = 6. Experimenters were blinded while acquiring data and during analysis.

Statistics

Compressive moduli data were assessed using one-way ANOVA followed by a Bonferroni's correction to determine significance ($p<0.01$) while minimizing type 1 error. Experimenters were blinded to hydrogel composition while acquiring the data and during analysis.

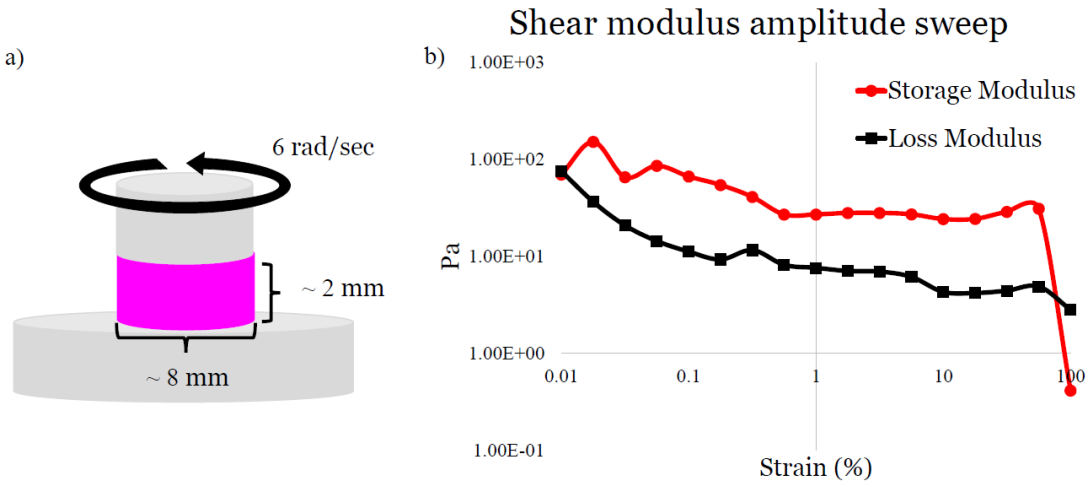

Figure 2.2: Shear modulus.
(a) To assess the response of hydrogels to shear stress, an amplitude sweep was performed on hydrogels of ~8 mm diameter and ~2 mm height using an Anton Paar rheometer. (b) The amplitude sweep was performed from 0.01%-100% strain to determine the linear viscoelastic range of strain, which in this case is from 1%-5.6%. A strain within this range was then chosen to perform the frequency sweep to assess the hydrogel shear moduli, which is comprised of two components, the storage modulus and the loss modulus. The storage modulus describes the elastic component of the hydrogel response whereas the loss modulus describes the viscous component of the response.

Gelation kinetics

Protein absorbance at 405 nm increases as crosslinking increases and hydrogel gelation occurs. This can be exploited to assess gelation kinetics by examining the increase and eventual plateau in absorbance during gelation. To assess time for gelation at 37°C, absorbance at 405 nm was assessed for 100 μL of pre-gel solution in a 96-well plate using a Synergy HT microplate reader (BioTek, Winooski, VT). Solutions were transferred from ice immediately to the pre-warmed (37°C) plate reader. Absorbance readings were recorded every 2 minutes for 50 minutes. A solution of DMEM+HEPES, diluted in PBS at the same concentration as in the hydrogels, was used as a negative control. Data was normalized to the initial readings following Equation 1, where A is the absorbance, A_0 describes the initial absorbance, and A_{max} describes the maximum absorbance, or steady state value.

$$Normalized\ Absorbance = \frac{A - A_0}{(A_{max} - A_0)} \qquad \textbf{Eqn. 1}$$

Statistics

Normalized absorbance readings were compared between groups using a one-way ANOVA assessment followed by Bonferroni's correction to assess between-group differences and minimize type 1 error with multiple comparisons. Experimenters were blinded to hydrogel composition during testing and analysis.

Hyaluronic acid release

Hyaluronic acid is not crosslinked into the hydrogel and therefore is expected to diffuse away from the hydrogel structure in vitro and in vivo. To assess the rate of release, hyaluronic acid was conjugated to fluorescein isothiocyanate (FITC) (American Peptide, Sunnyvale, CA, 316846) using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma-Aldrich, St. Louis, MO, 25952) - N-hydroxysuccinimide (Sigma-Aldrich, St. Louis, MO, 130672) (EDC - NHS) chemistry. FITC was chosen based on its small size, especially in comparison to hyaluronic acid, so the conjugation should not alter diffusion kinetics of this component. Briefly, 0.08 mg/mL FITC, 0.75 mg/mL EDC, and 0.78 mg/mL NHS was added to 10 mg/mL hyaluronic acid in deionized water and stirred on a stir plate for 4 hours at room temperature. This solution was then quickly transferred to a 10,000 kDa dialysis cassette and dialyzed for 48 hours in an excess of distilled water, with the distilled water replaced every 12 hours. Following purification via dialysis, the solution was transferred to a 50 mL conical tube and lyophilized until dry (~48 hours). This solid was stored at -20°C until being used in place of unconjugated hyaluronic acid during hydrogel synthesis.

30 μL Col HA Lam and Col HA hydrogels were placed in 120 μL of 37°C PBS in a 48 well plate, which was then placed in a humidified chamber in an oven at 37°C. Collagen hydrogels without HA (Col) were used to control for fluorescence associated with DMEM or uncrosslinked collagen release. The supernatant was removed every hour and stored for testing over the period of the experiment. A pilot experiment was performed over a 24 hour period at 3 hour intervals to determine a relevant experimental

length. The experiment was performed every hour for 9 hours to quantify HA release. Fluorescence of the supernatant was examined at 480±20 nm excitation and 518±20 nm emission to identify FITC-conjugated hyaluronic acid using a Synergy HT microplate reader (BioTek, Winooski, VT). Fluorescence was measured from below the plate to minimize error associated with long measurement distances. A standard curve of FITC-conjugated HA from 0 - 1 mg/mL in PBS was used to determine concentration upon release. Each composition of hydrogel containing hyaluronic acid was also dissociated in PBS and measured for total fluorescence to determine maximum HA content.

Statistics

All values are represented with mean ± standard deviation.

Mechanical characterization of hydrogels results

Assessing the mechanical properties of these hydrogels can provide insight to the cellular environment in culture and in vivo after transplantation. Recent literature suggests that mimicking native tissue is important to encourage directed differentiation (see Chapter 2), therefore mechanical characteristics of all hydrogel compositions used here were compared to compressive moduli of native neural tissue reported in literature.

The ultimate goal of this project is to create an injectable hydrogel that promotes oligodendrocyte differentiation, which is expected to stimulate axonal regeneration in vivo. Mechanical properties of the hydrogels play a key role in this directed differentiation. NPC differentiation into oligodendrocytes has been shown to occur in soft materials when the compressive modulus is 0.1 - 1 kPa [28], and the storage modulus is

near 100 Pa [28, 145]. In addition, general axonal regeneration is improved in materials with modulus from 0.4-5 kPa [28, 145].

Compressive moduli

The compressive modulus of each hydrogel composition was measured to assess mechanical properties of the hydrogels. To mimic the native tissue, these materials should be relatively soft (0.1-1 kPa) [28, 134, 145, 149-151]. Neonatal brain tissue has been reported to have a compressive modulus between 0.1 and 2.6 kPa [28, 134]. The goal of this project is to develop a material to mimic native tissues, therefore, the materials for this project were optimized to have compressive moduli from 0.4 - 2 kPa by adjusting collagen concentration.

Collagen alone (Col) hydrogels exhibited the highest modulus at 1.82 kPa, while the three-component hydrogels have the lowest modulus at 0.57 kPa (**Fig. 2.3**). These moduli are within range of developing central nervous system tissue, as indicated by the dashed line in **Figure 2.3**. It has been proposed that oligodendrocytes respond most favorably to compressive moduli 0.1 - 1 kPa [28], therefore, these hydrogels, particularly the three component hydrogel, may provide optimal conditions for the desired cell type.

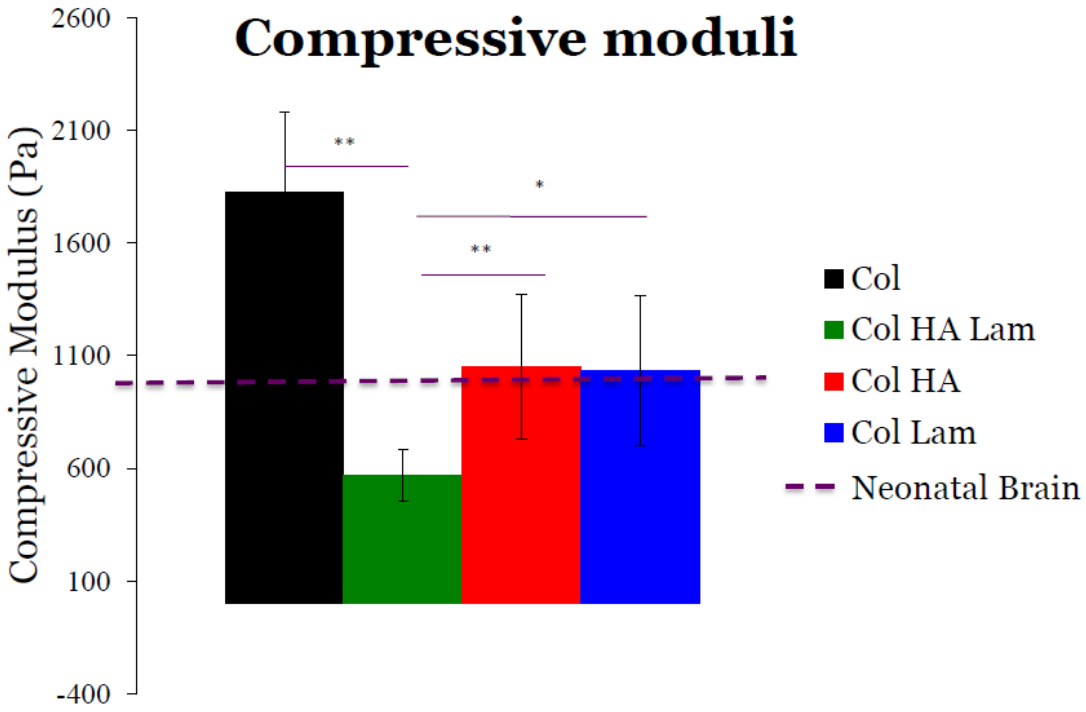

Figure 2.3 Compressive moduli of the four hydrogel compositions.
The collagen alone hydrogel has the highest compressive modulus (~1800 Pa), whereas the hydrogels with other components added (either HA or laminin) have lower moduli (~1200 kPa). The hydrogel with three components has the lowest compressive modulus (~ 570 Pa). Interactions between components may cause a decrease in compressive modulus by interfering with crosslinking by binding to the crosslinking component (in this case collage) near the crosslink site or by introducing a higher hydrophilicity, thus preventing as tight of crosslinks in a hydrated environment. The compressive moduli of these hydrogels are softer than neonatal brain tissue compressive modulus (dashed line (1)) and in range of the expected moduli that will provide the most optimal stimulus for oligodendrocyte maturation (~0.4-1 kPa) (1). Note: n=15/group, *=$p<0.05$, **=$p<0.01$, error bars are ± standard error of the mean.

Shear moduli

Shear moduli were measured via rheology. To mimic native CNS ECM, these materials should be soft (20 - 100 Pa Storage modulus [28, 145]. The storage and loss moduli of the Col Lam hydrogel were highest (60.8 Pa, and 7.9 Pa for storage and loss moduli, respectively), compared to other hydrogels, whereas the Col HA hydrogel exhibited the lowest moduli (29.5 Pa and 5.5 Pa) (**Fig. 2.4, Table 2.2**). The Col HA Lam hydrogels exhibited moduli in the middle of the range achieved, with a storage modulus of 42.6 Pa and a loss modulus of 6.4 Pa. All of these components altered the shear moduli of a collagen base hydrogel, which exhibited storage and loss moduli of 33.9 Pa and 7.1 Pa, respectively, in a predictive way based on the additional components of the hydrogel. Proteins respond in a more elastic manner to shear stress than glycosaminoglycans. Specifically, HA is a shear thinning glycosaminoglycan, so the response to shear stress may be more viscous rather than elastic in hydrogels with high HA content. All of the hydrogels tested followed the expected pattern of exhibiting a higher storage modulus than loss modulus, implying they exhibit material properties more similar to an elastic solid than a viscous liquid under shear stress.

To mimic the native ECM environment most appropriately for the applications, the rate of cell growth and migration needs to be considered. Assessment of shear modulus at low frequencies is appropriate to determine the cellular environment because cells tend to grow and migrate slowly, so they would experience the hydrogel response to slow oscillatory shear forces, under which conditions, the storage modulus of brain tissue

was measured as near 100 Pa [145, 152, 153]. A frequency sweep allows assessment of material response over a range of behaviors, including cell growth and migration as well as frequencies associated with the systemic environment (6 rad/sec). The data reveal that the behavior of these hydrogels is similar to other soft hydrogels, with a sudden dip in the loss modulus above 10 rad/sec [145] (**Fig. 2.4**).

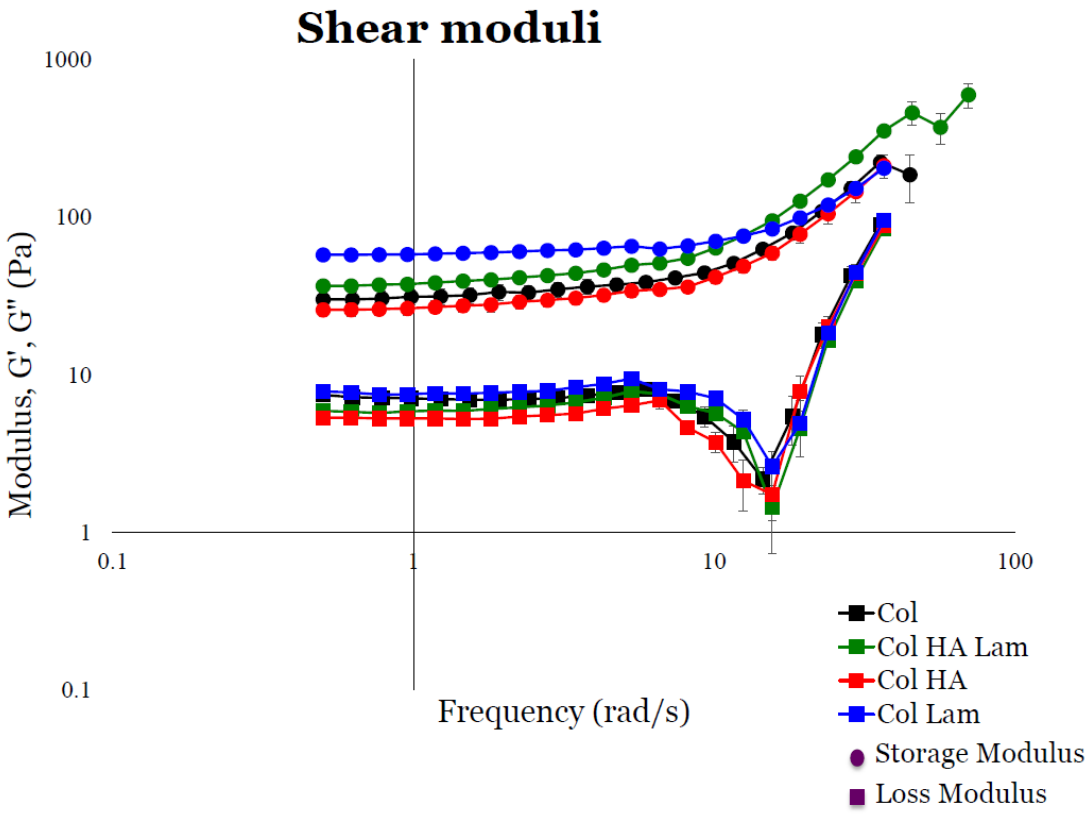

Figure 2.4: Shear moduli.
Storage and loss moduli were measured for each hydrogel type. A representative plot of each hydrogel type is provided here. Collagen HA hydrogels have the lowest moduli, and Collagen Laminin hydrogels have the highest moduli. G' below 200 Pa is in range of brain tissue (Levental) (Error bars are ± standard error of the mean. Note: n=6/group. Error bars are ± standard error of the mean. This experiment was repeated twice.

Table 2.2: Storage and loss moduli ± standard error of the mean

Hydrogel Type	Storage Modulus (Pa, ± SEM)	Loss Modulus (Pa, ± SEM)
Col	33.9±0.991	7.1±0.156
Col HA Lam	42.6±1.568	6.4±0.181
Col HA	29.5±0.947	5.5±0.150
Col Lam	60.8±0.765	7.9±0.145

Gelation kinetics results

Because this project involves an injectable hydrogel, understanding gelation kinetics is important to evaluate the efficacy of this material for the proposed application. Gelation should occur slow enough to enable injection into the injured spinal cord without a sudden pressure increase or damage to the cord caused by the injection. However, in culture, the hydrogels are gelled prior to the addition of medium, therefore, rapid gelation is ideal so cells survive the encapsulation process without nutrients. The four hydrogel compositions exhibited different gelation times as determined from solution turbidity. Hydrogels containing laminin tended to gel slower (~26 minutes) than those without laminin (~18 minutes). HA did not appear to have an effect on gelation time. All hydrogels were completely gelled in 30 minutes at 37^{o}C (**Fig. 2.5**). This timeframe is fast enough to allow cells to survive the gelation process yet slow enough to enable the experimenter to inject the hydrogel over 2 minutes during in vivo transplantation without concern of increased viscosity or internal pressure. Additional time is accounted for to align the needle with the injection site, therefore the ideal range is from 8-30 minutes for gelation. These date reveal some noise at the early timepoints indicating an interaction of the measurement with condensation on the plate. Additionally, when laminin is present, a decrease in the absorbance occurs prior to a sudden increase in absorbance, possibly due to the interaction of laminin and collagen during the gelation process [6, 138, 146].

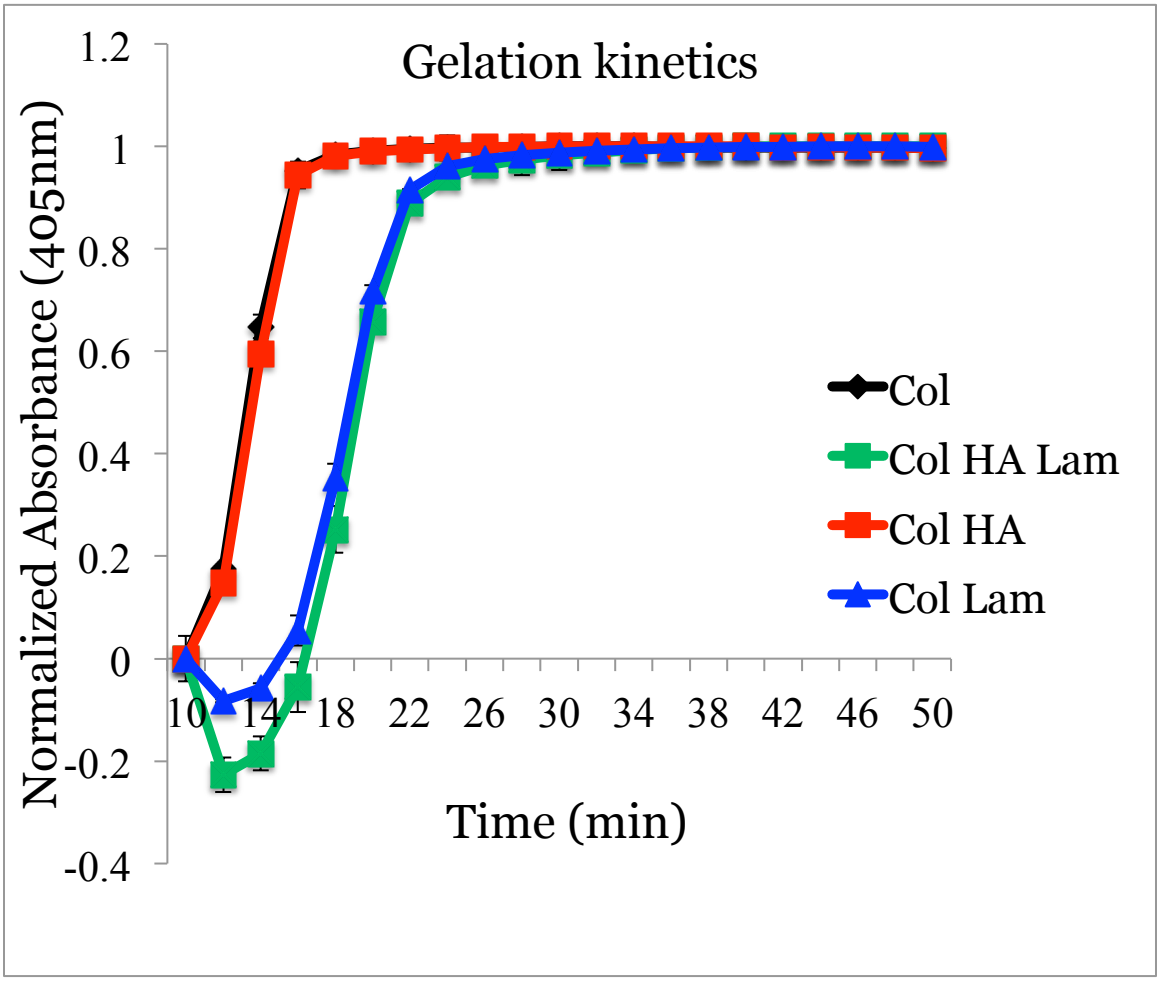

Figure 2.5: Gelation kinetics.
Gelation kinetics were assessed by examining the absorbance of 512 nm wavelength light through the hydrogel. As proteins crosslink, the absorbance through them at this wavelength increases. In a protein hydrogel, the measured values should equilibrate once the hydrogel reaches a gelled state. In all hydrogel types, the absorbance equilibrated within 30 minutes. Error bars represent ± standard deviation.

Hyaluronic acid release results

HA is not crosslinked into the hydrogels; therefore, it was hypothesized that the HA would diffuse from the hydrogel during in vitro culture and in vivo after transplantation. As predicted, complete HA release was observed by 9 hours in culture with PBS at 37 ºC from each of the hydrogels with HA (Col HA and Col HA Lam) (**Fig. 2.6**). It is important to release this component to allow oligodendrocytes to mature as neurites extend to allow the interaction between neurons and oligodendrocytes. Additionally, this component is shown to minimize scar formation in vivo; therefore, its short-term presence early after transplantation is beneficial.

Specific Aim 1 Summary

Four hydrogel types were examined for their compressive and shear moduli as well as gelation kinetics and HA release. To mimic native neural tissue, compressive and storage moduli were optimized to 0.4 - 2 kPa and 20 - 150 Pa, respectively. Additionally, HA was not crosslinked into the hydrogels and was shown to diffuse out of the hydrogels in 9 hours. The rapid release of this component ensures short term signaling to the cells in culture and had profound effects on differentiation outcomes.

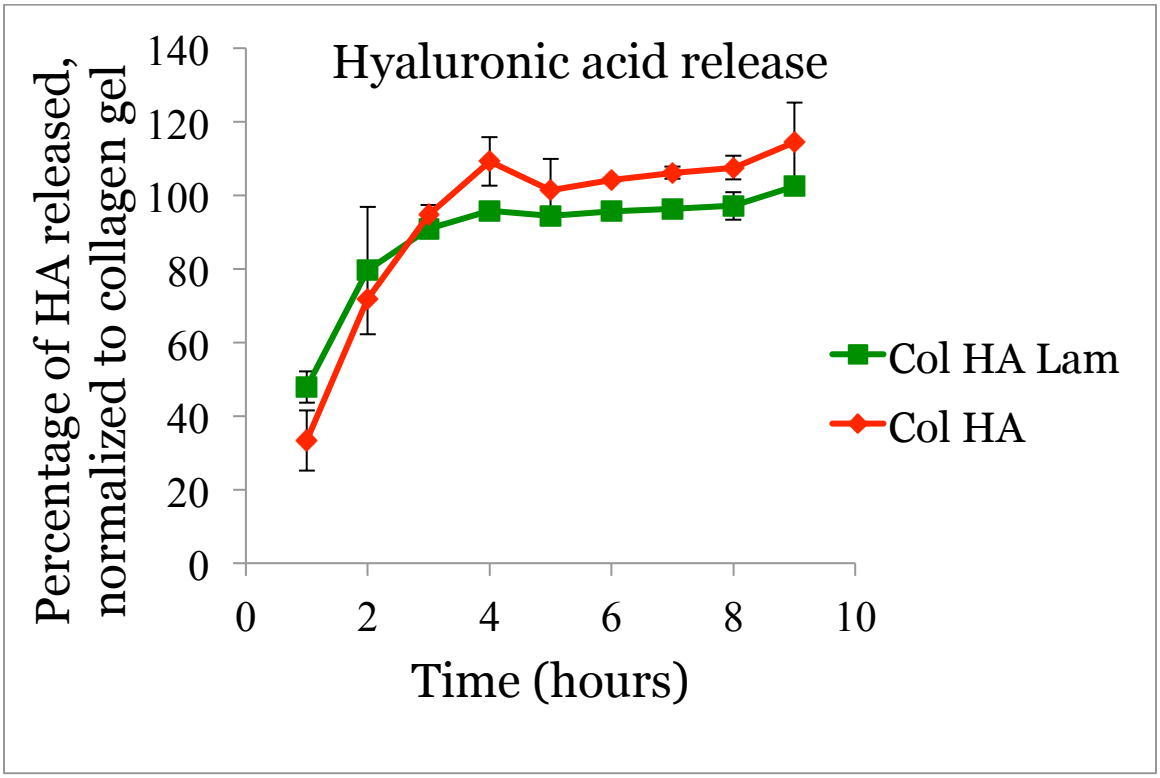

Figure 2.6. HA release from hydrogels.
HA is not crosslinked into the hydrogel, therefore, it is expected to diffuse from the injected area when introduced to the in vitro and in vivo environments. To examine HA release from the hydrogels, HA was conjugated to FITC and hydrogel supernatant was collected every hour for 9 hours. Fluorescence of the supernatant was measured and normalized to fluorescence of supernatant from collagen alone gels in PBS. Concentrations of HA released to the supernatant was calculated based on a standard curve of concentration of HA. HA was released by 9 hours in each hydrogel type that contained HA. Note: n=6/group, this experiment was repeated twice.

Chapter 3: In Vitro Cell Differentiation

SPECIFIC AIM 2: EXAMINE THE DIFFERENTIATION OF NEURAL PROGENITOR CELLS TOWARD OLIGODENDROCYTES EMBEDDED IN DIFFERENT HYDROGEL COMPOSITIONS.

Oligodendrocytes are beneficial to the regenerating central nervous system as they provide support to growing axons. By myelinating growing axons and remyelinating damaged axons, transplanted oligodendrocytes can be a powerful tool to assist in functional recovery. Oligodendrocytes differentiate in the neonatal period of development, which this biomaterial scaffold is designed to mimic.

Cell isolation and neural progenitor cell culture

All animal work was performed in accordance with the Institutional Animal Care and Use Committee at The University of Texas, Austin or the University of Florida. The spinal cords of E12 mice (C57BL6, Charles River) or E14-15 rats (Sprague-Dawley, Charles River) were isolated into Hank's Buffered Salt Solution. Spinal cords were placed in DMEM F12 media with N2 supplement and a penicillin/ampicillin/streptomycin solution and dissociated mechanically by pipetting with a fire polished glass Pasteur pipette. About $1X10^6$ cells were plated in a 10 cm tissue culture dish and cultured as free-floating cell aggregates or "neurospheres" in the same medium supplemented with 20 ng/mL basic fibroblast growth factor added every two days. After 7-14 days, the neurospheres were dissociated by pipetting with fire polished Pasteur pipettes. Cells were then split to 500,000 cells/plate and expanded for up to five passages. This culture technique is widely used to expand neural progenitor cells without inducing differentiation [154-156]. To verify that NPCs did not differentiate during

expansion, neurospheres were sectioned and stained for nestin, a marker for immature neural progenitor cells. Neurospheres exhibited minimal signs of differentiation (data not shown); however, for this study, only primary cells or cells from passages one or two were used. Hydrogels were seeded with cells at 100,000 cells/ 30 μL hydrogel, either immediately after isolation or after the first or second passage.

Methodology for immunostaining and imaging for in vitro differentiation

Cells encapsulated in hydrogels were cultured for five days, fixed in 4% paraformaldehyde for 20 minutes and prepared for immunostaining. Hydrogels were separated into two groups, (1) those to be stained for intracellular components and (2) those to be stained for membrane-bound components. Group 1 was prepared with a blocking buffer that contained 5% goat serum (Sigma-Aldrich, St. Louis, MO, G9023), and 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO, 93443) in PBS. Group 2 was prepared with a blocking buffer that contained only 5% goat serum in PBS as membrane permeabilization by Triton X-100 was unnecessary and would disrupt membrane-bound protein localization and appearance. For each group, nonspecific binding was blocked in blocking buffer 1 hour at room temperature. In group 1, primary antibodies against β-III tubulin (to detect neurons, 1:500, mouse polyclonal Abcam, Cambridge, England, ab7751) and glial fibrillary acidic protein (GFAP) (to detect astrocytes, 1:500, rabbit polyclonal, Abcam, Cambridge, England, ab7260) were used and cell nuclei were counterstained with DAPI. In group 2, primary antibodies against NG2 (to detect oligodendrocytes, rabbit polyclonal, US Biological, Salem, MA, C5067) and O4

(Millipore, Darmstadt, Germany, MAB345) were used. In both groups, nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI).

Fluorescence images were acquired using a Zeiss AxioImager Z2 (Zeiss, Jena, Germany) with a 20X objective. Image stacks were reconstructed into 3D projections using Zen software (Zeiss, Jena, Germany). Six images throughout each hydrogel were acquired on the AxioImager to assess cell differentiation. Each image was analyzed by manually counting the number of DAPI stained nuclei present using ImageJ software. Cells stained for each component were counted similarly; each image was overlayed with DAPI to minimize the possibility of double counting if cell membranes were not intact. Cells were only counted if the nucleus and the cell body were identified as colocalized. Two independent, blinded experimenters counted cells, and their scores were compared to minimize bias.

Statistics

A one-way ANOVA followed by a Bonferroni assessment was used to determine statistical significance ($p<0.01$) of cell type in different hydrogel compositions.

Mouse cell culture results

Mouse cell differentiation was examined to identify the hydrogel composition with the most efficient oligodendrocyte differentiation. All cell types (astrocytes, neurons, and oligodendrocytes) were present in all hydrogel compositions (**Fig. 3.1**), however, the differentiation profiles were different in each hydrogel composition. The three-component hydrogel (Col-HA-Lam) had the highest oligodendrocyte differentiation

efficiency with 59.1% oligodendrocyte differentiation. The hydrogel that produced the next highest percentage of oligodendrocytes was the collagen-laminin (Col Lam) and collagen-HA (Col HA) hydrogels with 27.2% and 26.7% NG2+ cell presence, respectively. Collagen hydrogels performed least efficiently for the 3D culture, with 1.5% NG2+ cell differentiation. **Figure 3.2** shows the differentiation profiles in different hydrogel compositions, revealing the percentages of oligodendrocytes, astrocytes, and neurons. Cells were stained using anti-NG2 and anti-O4 antibodies to identify oligodendrocytes, anti-GFAP antibody to identify astrocytes, and anti-β-III tubulin antibody to identify neurons. Collagen alone (Col) hydrogels had the highest percentage of astrocyte differentiation, while collagen-HA (Col HA) hydrogels had the highest percentage of neurons present in the hydrogels. The three-component hydrogel increased differentiation of oligodendrocytes and neurons as compared to the collagen alone hydrogel. These results confirmed the three-component hydrogel's applicability as a transplantation vehicle for neural progenitor cells.

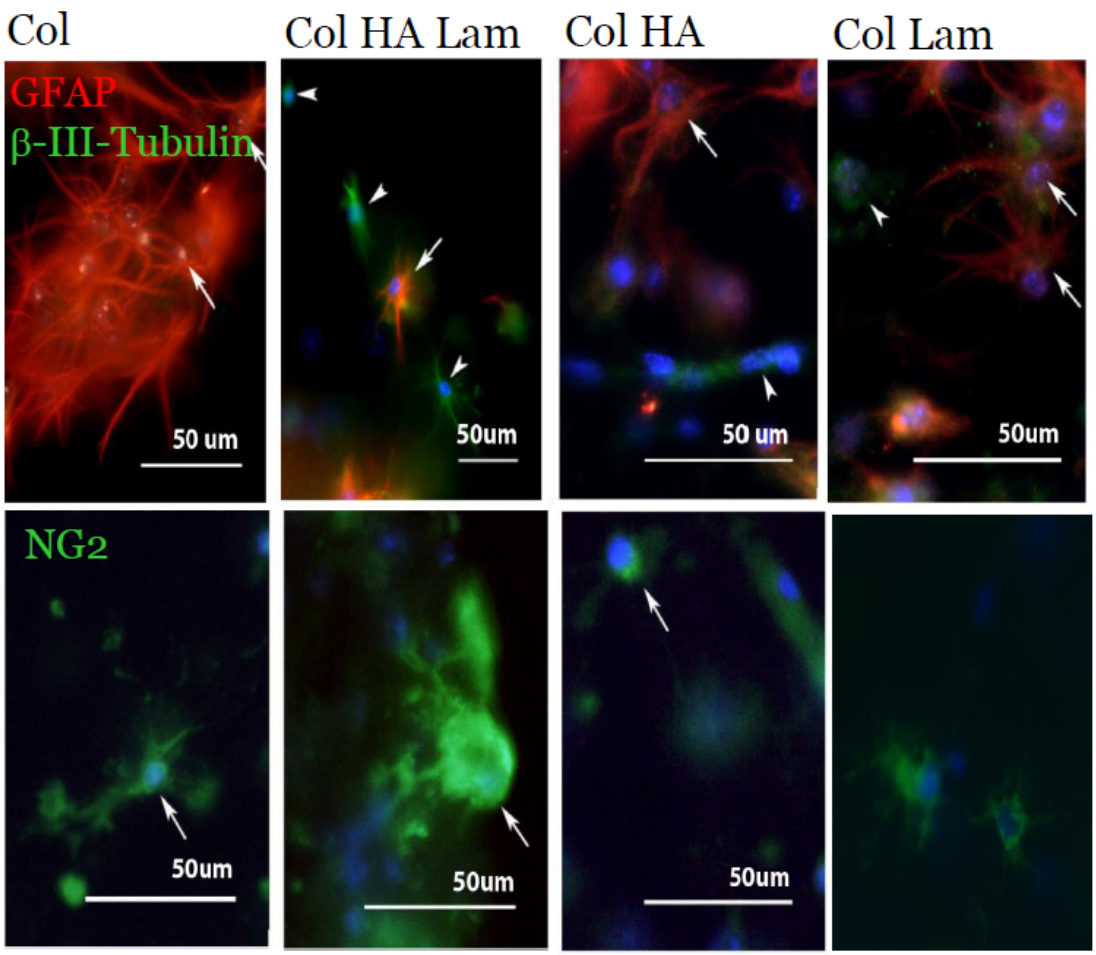

Figure 3.1: Mouse cells cultured in four hydrogel compositions examined with immunocytochemistry.
Cells were stained with anti-GFAP (red, top) to identify astrocytes, anti-β-III tubulin (green, top) to identify neurons, and anti-NG2 (green, bottom), to identify oligodendrocytes. All cell types were present in each of the four hydrogel compositions. However, the morphology and differentiation profiles were different in each.

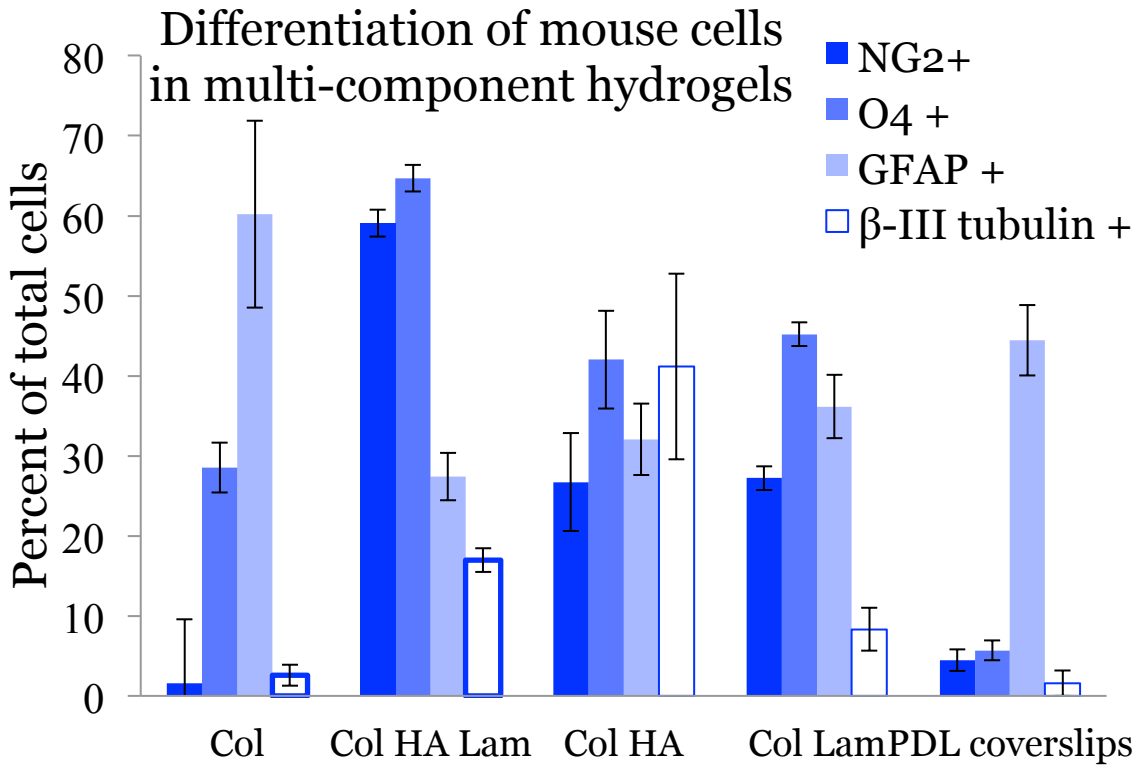

Figure 3.2: Mouse cell differentiation in hydrogels.
E11.2-E14 mouse spinal progenitor cells were cultured in hydrogels for 5 days with DMEM F12 medium supplemented with 1% fetal bovine serum. No specific differentiation signals were provided. Oligodendrocytes (O4 and NG2) differentiated most optimally in Col HA Lam hydrogels, whereas Col gels promoted astrocyte differentiation. The cells were responding to the ECM components as well as the mechanical properties of the hydrogels. The phenotypic response of the cells is in response to the ECM components present and the mechanical properties of the hydrogel. Note: n=12/group, this experiment was repeated three times.

Rat cell culture results

Rat cell differentiation was examined similarly to mouse cell differentiation. Similar trends were present in rat cell culture as were seen in mouse cell culture (**Fig. 3.3**). Significantly higher values ($p<0.05$) were seen for oligodendrocyte differentiation in the three-component hydrogel for rat (66.75%) compared to mouse (59.1%) cells. Lower differentiation percentages of neurons were observed in Col HA hydrogels with rat cells (26.9%) compared to mouse (41.1%) cells ($p<0.01$). These differentiation profiles are promising to support the hypothesis that ECM composition and mechanical properties of the environment affect neural progenitor cell differentiation.

Specific Aim 2 Summary

The extracellular matrix components present, gelation kinetics, mechanical characteristics, and HA release all contribute to cellular differentiation in culture. Softer hydrogels, with compressive modulus of 0.57 kPa, storage modulus of 42.6 Pa, and loss modulus of 6.4 Pa optimally encourages differentiation of NPCs toward oligodendrocytes. These hydrogels provide cues to NPCs to direct their differentiation toward the three cell types present in the CNS; neurons, astrocytes, and oligodendrocytes. In an attempt to remyelinate axons that have been demyelinated after injury, hydrogels were optimized to direct the differentiation of cultured cells toward oligodendrocytes. Ultimately, these hydrogels were designed to act as a delivery vehicle for NPCs to the injury area after spinal cord injury and to provide cues to the cells after transplantation. The three-component hydrogel was selected for transplantation into the rat spinal cord injury model.

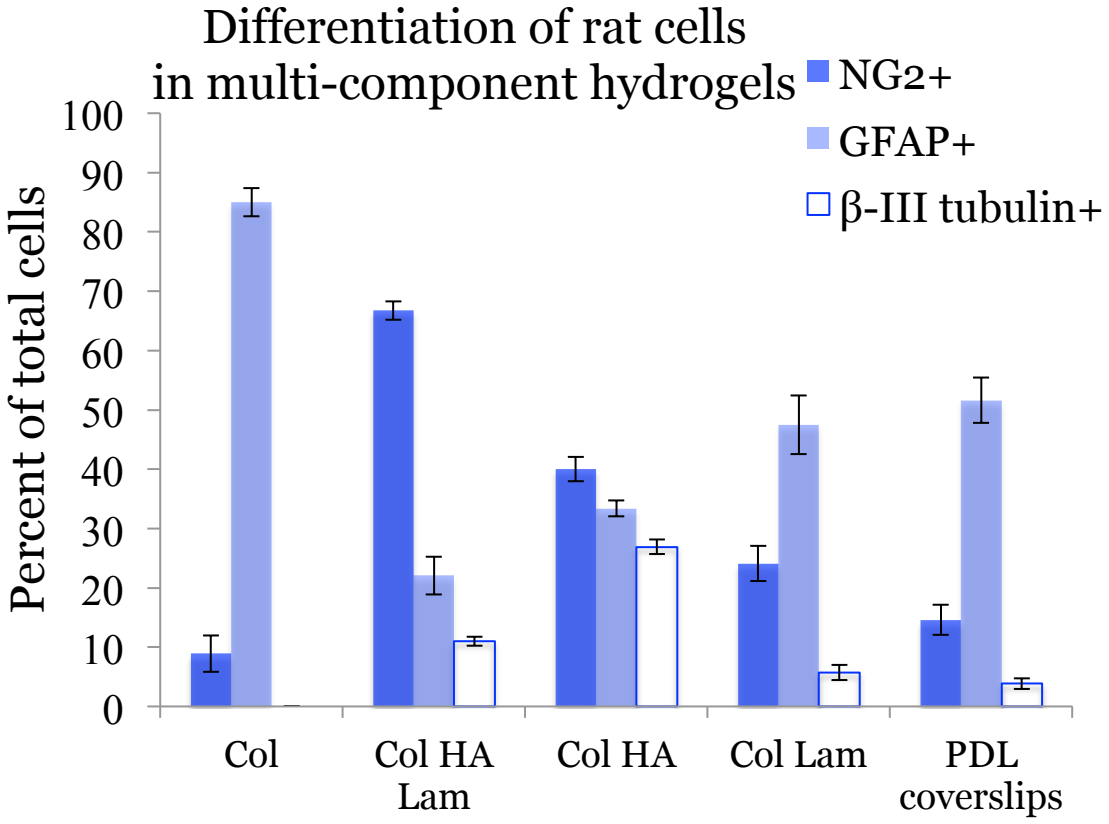

Figure 3.3: Rat cell differentiation in hydrogels.
E14-E15 rat spinal progenitor cells were cultured in hydrogels for 5 days in DMEM F12 medium supplemented with 1% fetal bovine serum. No specific differentiation signals were provided. Similar to mouse cell cultures, oligodendrocytes (NG2) differentiated most efficiently in Col HA Lam hydrogels, whereas Col gels promoted astrocyte differentiation. The cells were responding to the ECM components as well as the mechanical properties of the hydrogels. Note: n=10/group, this experiment was repeated twice.

Chapter 4: In Vivo Assessment

SPECIFIC AIM 3: IMPLEMENT AN INJECTABLE HYDROGEL-CELL SYSTEM INTO AN IN VIVO MODEL TO ASSESS FUNCTIONAL AND ANATOMICAL RECOVERY AFTER SCI.

Spinal cord injury

Model development.

Contusion spinal cord injury was performed on female rats ages 8-10 weeks. This model is clinically relevant and an ideal hydrogel transplantation model: A cavity forms within the spinal cord after contusion injury providing a distinct injection location that is surrounded by spinal cord tissue, preventing diffusion of the pre-gel solution prior to gelation.

Though this model has been previously developed and reported in literature, for the purpose of this project, specific verification and characterization was performed. Lesion volume analysis was assessed after application of a known contusion force. Five animals were euthanized one week post injury by trans-cardiac perfusion as described below. 12 μm cross sections were stained with standard cresyl violet staining. Analysis was performed every 120 μm to assess lesion size. The Cavalieri method [157] was used to estimate lesion volume to determine the appropriate hydrogel volume for transplantation. Briefly, using ImageJ, area of the lesion was determined on each stained section, and lesion volume was estimated using a rectangular Cavalieri's method; this method expands the area of each section to a volume by multiplying the section area by the distance between sections (**Fig. 4.1**). One week after contusion spinal cord injury, the lesion volume was determined to be 0.62 ± 0.03 μL.

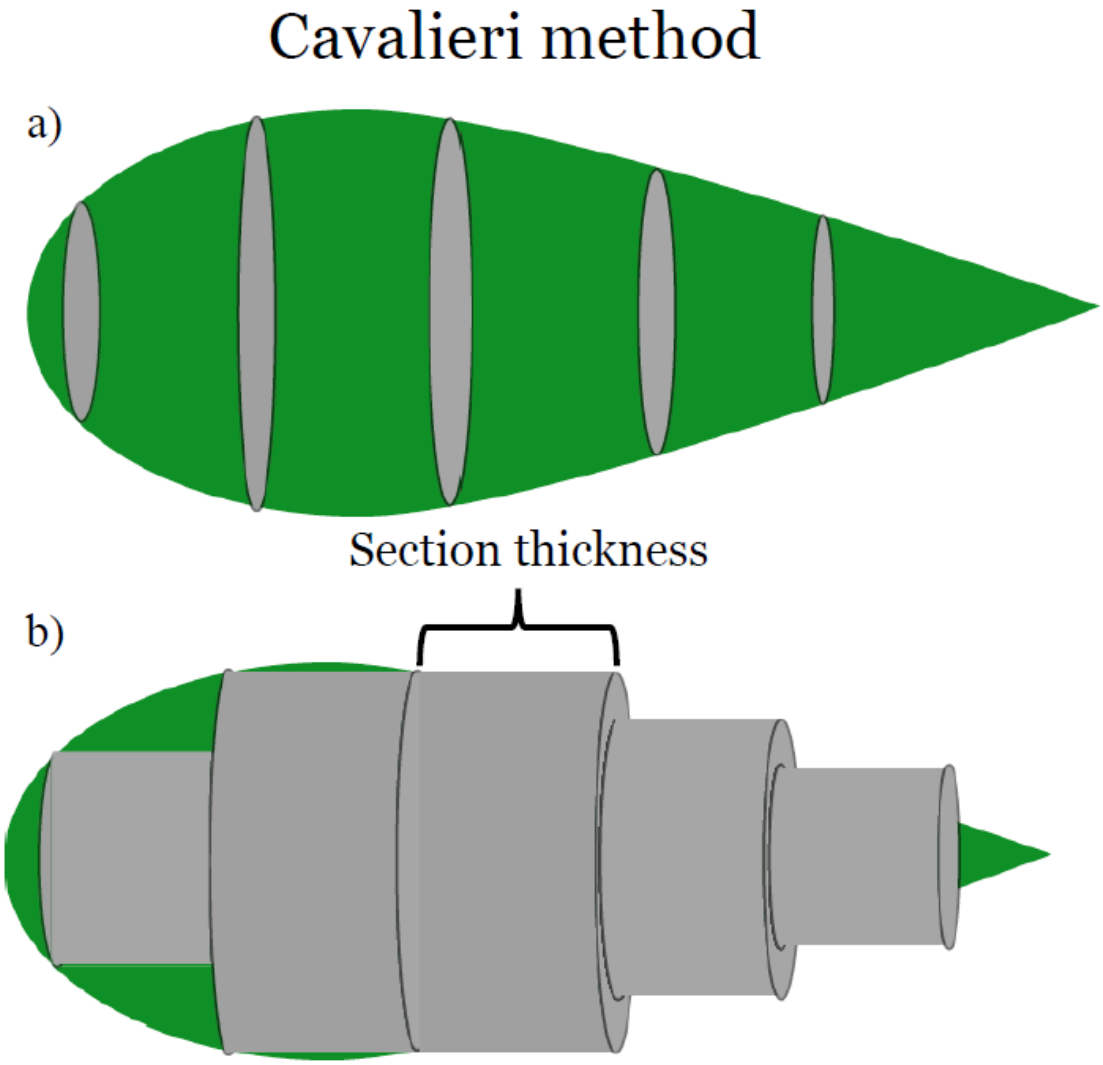

Figure 4.1: Cavalieri method.
The Cavalieri method is a robust method to estimate the volume of unknown geometries. To assess this volume, sections are taken through a 3D object (a), in the case of this dissertation the lesion within the spinal cord, and the volume is projected through the next section. This leaves a volume assessment of consecutive solids with unchanging geometry throughout them. In this figure this is a series of consecutive cylinders (b), each of different diameter. This volume assessment was found to be the most optimal for CNS volume estimation (cite).

Surgical methodology

All animal work was performed in accordance with the Institutional Animal Care and Use Committee at The University of Texas, Austin or the University of Florida. Adult female rats (Sprague Dawley; Charles River), 8-10 weeks old, were anesthetized using isoflurane vapor (3% to induce and 2% for maintenance), and ophthalmic ointment was placed on the rats' eyes to prevent drying during surgery. The surgical area from just below the scapula to between the ears was shaved and cleaned using chlorhexidine scrub and sterile saline to prepare the animals for surgery. A total of 24 animals were used for a two week experiment, and 40 animals were used for a 6 week experiment. Four groups (n=6) were included in the two week experiment, and five groups (n=8) were included in the six week experiment, as outlined in **Table 4.1**. A fifth group (laminectomy) provided a behavioral control without injury. The animals were subjected to surgery up to and including a laminectomy (removal of the dorsal portion of the vertebra) but did not receive any injury to the spinal cord.

Anesthetized animals were transferred to the surgical table where they were kept under isoflurane vapor anesthesia for the duration of the surgery. A dorsal laminectomy was performed at the fourth cervical vertebra (C4) to expose the underlying spinal cord. Forceps on the Infinite Horizon Impactor (Precision Systems and Instrumentation, Virginia) were used to stabilize the vertebrae of C5 and C3. Animals to receive a contusion were placed under the Infinite Horizons Impactor fitted with the mouse impactor head (1.3 mm diameter) and an impact force of 150 kDynes was applied laterally on the left to induce a contusion injury. The wounds were then closed in layers

(muscle and overlying skin) using 5-0 absorbable vicryl sutures and 5-0 monofilament nylon sutures, respectively.

Pain associated with surgery was managed using buprenorphine hydrochloride (0.03 mg/kg, Patterson Veterinary, Devens, MA, 07-850-2280) administered subcutaneously immediately prior to surgery and every 8-12 hours following surgery for 48 hours. The antibiotic gentamicin sulfate (4-5 mg/kg, Patterson Veterinary, Devens, MA, 07-805-7791) was administered subcutaneously every 8-12 hours for 5 days starting one day prior to surgery and repeated if signs of infection were detected. Lactated Ringer's solution (5 mL) was subcutaneously administered immediately following surgery and at later stages as animals showed signs of dehydration. This type of unilateral contusion spinal cord injury induced limited mobility with one forelimb and one hindlimb immediately following surgery. Animals regained hindlimb function to normal levels within two days after injury. All animals were examined to ensure their ability to access food and water readily during their recovery period of two weeks. All animals were monitored three times daily for five days after injury, and twice daily for the remainder of the two week recovery period. Animals were weighed daily; if they lost more than 10% of their pre-surgery body weight, dietary supplements such as NutriCal (Patterson Veterinary, Devens, MA, 07-836-0262) or DietGel Boost (Clear H2O, Portland, ME 72-04-5022) were administered to supplement their diet. DietGel was administered for the first two days after injury and after as needed based on weight loss. This type of injury did not affect bladder function, however voluntary bladder voiding was assessed twice daily for the first three days after surgery.

Table 4.1: In vivo experimentation groups.

Experimental Group	Group Size	
	Two Week	Six Week
Hydrogel + Cells	6	8
Hydrogel Alone	6	8
Media + Cells	6	8
Media Alone	6	8
Laminectomy	-	8

Cell and hydrogel transplantation methodology

Prior to treatment transplantation, animals were separated into experimental groups based on behavioral scores acquired with the forelimb step-alternation test. One week post injury, animals were weighed and prepared for surgery as described above. The previous surgical site was reopened by removing sutures in the skin and gently pulling the skin apart. Muscle layers were separated similarly, and the spinal cord was re-exposed to reveal the injury area. The tip of a 34 gauge needle on a NanoFil microinjection system (World Precision Instruments, Sarasota, FL) attached to a stereotactic stand was inserted into the spinal cord in the injury area approximately 50 μm deep. A 0.6 μL solution of either Col HA Lam hydrogel solution or media with or without NPCs (passage 1) was slowly injected over 2 minutes into the cavity area. For the groups with cells, 20,000 cells were transplanted into the lesion cavity. The wounds were then closed in layers (muscle and overlying skin) using 5-0 absorbable vicryl sutures and 5-0 monofilament nylon sutures, respectively.

All pain management and post-operative care was repeated similar to after the initial spinal cord injury surgery with one exception immediately following transplantation surgery. Meloxicam (1 mg/kg, Patterson Veterinary, Devens, MA, 07-890-7338) was also administered to animals for additional pain management.

Behavioral assessment methodology

Prior to surgery, a total of 3 weeks of handling and pre-testing was performed to acclimate the rats to being handled and to ensure that the animals exhibited normal behavior prior to surgery. Experimenters were blinded to animal group throughout

behavioral assessment. All behavioral tests were performed twice prior to surgery, immediately prior to transplantation, and once weekly following transplantation for up to 6 weeks. Between-experimenter variation was minimized by maintaining the same experimenter for alternation, postural instability, and placing tests and utilizing two experimenters for the pasta, cylinder, and grooming tests.

Postural instability test (PIT)

To perform this test, animals had to be relaxed and comfortable in the experimenter's hands; any struggle during a trial would disqualify that trial. Rats were held in an almost vertical position with only their forelimbs in contact with the tabletop, similar to a wheelbarrow type position, while their hindlimbs were allowed to hang free. This test was performed on P220 sandpaper to prevent slipping, bracing, or dragging of the forelimbs during testing. Lines were drawn on the sandpaper every 1 cm to facilitate scoring of distance during stepping. The experimenter first lined the rats' noses up with the zero line as observed from above. Each rat was moved by the experimenter to shift the rats' center of gravity forward over the forelimbs, stimulating the rat to take a step to regain its balance. The new position of the nose upon stepping was measured as the distance needed to trigger a step. Each forelimb was tested independently by lightly restraining the opposite forelimb during testing. Four successful trials on each forelimb were performed each testing period.

Forelimb step-alternation test

This test was previously developed in collaboration with fellow researchers in Dr. Christine Schmidt's lab and with Dr. Timothy Schallert in the Psychology and Neuroscience departments at The University of Texas [73, 105]. A modification of the PIT, this test assesses the animals' ability to alternate forelimbs during a forward stepping motion. This test involves both forelimbs touching the tabletop initially in a comfortable stance, as determined by allowing the forelimbs to reach the table while the rat was held in an upright, wheelbarrow position. This allows determination of the alternation status of the animals; whether they are alternators (alternate the use of their forelimbs) or non-alternators (fail to alternate use of their forelimbs) can yield information regarding lesion location and size. While the experimenter moved the rat forward over their forepaws, first, step paw preference was noted (left or right), and the rats were observed for their ability to alternate steps. Four successful trials were performed each testing period.

Vibrissae-elicited forelimb placing test

A vibrissae (whisker)-elicited forelimb placing test was used to determine forelimb placing asymmetry, modified as previously described in our manuscript from 2012 [73]. The rats were held by their torsos, allowing their forelimbs and hindlimbs to hang free. The tail was also required to swing free: The score was not counted if the tail was used for support, nor if any of the limbs were used for support on any surface aside from the testing table. When instances of struggling stopped and muscle relaxation was achieved, the vibrissae on one side, and then the other, were stimulated by brushing them along a table top for ten trials on each side. A score of 0-4 was given based on the

targeted forelimb's placing response. Intact animals typically place each forelimb on the tabletop and receive a score of 4 because of accuracy of placement. A score of 0 was given when no response was seen. A score of 1 was given when the animal's limb showed movement but was insufficient to reach the table. A score of 2 was given if the animal's forepaw touched the table but not the top of the table, and a score of 3 was given if the animal's forepaw touched the tabletop with non-normal paw position. Average scores were calculated based on treatment group. Ten successful trials were measured each testing period.

Cylinder paw use preference test

The rats were rated live while being filmed in a transparent cylinder (20 cm diameter X 30 cm height) for 20 steps, but at most up to 2 minutes [73, 91]. Two experimenters independently scored each animal and compared results to minimize risk of bias. Three behaviors were scored during vertical exploration: (1) independent use of the left forelimb for weight bearing, (2) independent use of the right forelimb for weight bearing, and (3) use of both forelimbs simultaneously or rapidly alternating for weight bearing. In rare cases when experimenters were unable to distinguish which limbs were used, or whether simultaneously or independently used, the movement was not scored. Behaviors were scored as a percent use of each forelimb to total limb use, where half of the simultaneous use scores were allocated to each limb (Equation 4.1).

$$Paw\ use = 100 * \frac{independent + \frac{1}{2} simultaneous}{total} \qquad \textbf{Eqn. 4.1}$$

Pasta handling and eating

Rats were observed while eating 4 cm strands of dry pasta [73, 102, 103]. Paw preference was noted during each pasta eating session. The test was administered at approximately the same time each day. Two weeks prior to injury, animals were given the same type of dry pasta to prepare them for testing. The rats were observed beginning the week prior to surgery and weekly thereafter. The animals were tested with three pasta pieces each testing period. Each paw was scored independently for use. The paw was considered in use if the paw was placed on the pasta in a supportive resting position or if the paw gripped the pasta for more than half of the time during eating. The average time to begin using the ipsilesional paw was calculated for each group for the 75% of animals that recovered this function. The animals were evenly distributed throughout the groups.

4.1.1.1. Grooming test

Animals were wetted using a soft gauze sponge with clean water starting at their noses up to the center of their backs. Animals were observed while grooming themselves in a familiar environment (a clear cylinder similar to the one used for the paw preference test) and scored based on their ability to groom different levels of their body using each paw on a scale of 0 - 5 (modified from [87]). A score of 0 indicated that the animal did not lick their paw; a score of 1 was given if the animal licked their paw and brought it to their nose. A score of 2 was given if the rat groomed between their eyes and their nose, a score of 3 was given if the animal groomed above their eyes but below their ears, a score of 4 was given if the animal groomed their ears, and a score of 5 was given if the animal

was able to groom behind their ears to the back of their neck. Grooming score descriptions are pictorially represented in **Figure 4.2**.

Figure 4.2: Grooming.
Animals were observed while grooming and scored based on the level of function they expressed. Briefly, a score of 0 would imply that the animals did not lick their forepaw or touch it to any part of their face, a score of 1 describes the function of an animal that is able to lick their paw and touch their nose with that paw. A score of 2 implies that the animal can clean between their nose and eyes, whereas a score of 3 would describe the ability of an animal to groom above their eyes. An animal receiving a score of 4 should be able to clean its ears and a score of 5 describes an animal's ability to clean behind its ears and onto its upper back. This scoring system allowed us to examine the recovery of forelimb function.

Statistics

Behavioral data, with the exception of data obtained from the limb alternation and pasta eating tests, were analyzed by one-way ANOVA between groups (hydrogel + cells, media + cells, hydrogel alone, media alone, and laminectomy). Post-hoc analyses were performed when appropriate using the Bonferroni test (e.g. for PIT, vibrissae-elicited placing, cylinder preference test, and grooming test). All descriptive statistics are reported as mean ± standard error of the mean (SEM). The limb alternation test and pasta eating test present data in percentage of animals per group, thus statistical examination is limited.

Tissue harvest

At the end of the experimental periods, the animals were deeply anesthetized using an overdose of ketamine/xylazine. Trans-cardiac perfusion was performed using ice cold PBS (pH 7.4, ~200 mL) followed by cold 4% paraformaldehyde (~300 mL). The spinal cords were removed, post-fixed in 4% paraformaldehyde solution overnight at 4°C, and treated with 30% sucrose solution with 0.01% sodium azide prior to freezing. Sections were obtained using a cryostat (Leica, Wetzlar, Germany, CM1950) at 12 μm, thaw mounted onto gelatin-coated glass slides, and stored at -80°C.

Methodology for immunostaining and imaging for in vivo experiment

Tissue sections were dried on a slide warmer (Microscopes America, Cumming, GA, XL-2001) and a well was created on the slide using a hydrophobic pen. Sections were prepared for immunostaining in the same groups as the in vitro experiment (1) for cytoskeletal features and (2) for membrane features. Tissue sections were stained using

antibodies against (1) GFAP, b-III tubulin, nestin, bFGF receptor, and male-specific antigen UTY and (2) NG2, bFGF receptor, and UTY. UTY and bFGF receptor were used to identify transplanted cells. Additionally, some sections were stained for collagen I to identify the transplanted hydrogel. A description of each antibody and its targets can be found in **Table 4.2**.

Some sections were stained using contrast staining methods including Hematoxylin and Eosin (H&E) staining or cresyl violet staining. For H&E staining, sections were dehydrated in xylene and ethanol, then rehydrated in water. Sections were then dipped in Harris Hematoxylin solution (Sigma Aldrich, St. Louis MO, HHS-16-500ML) to stain nuclei, rinsed in water and placed in Scott's solution until they turned blue. Following that treatment, sections were rinsed again in water and dipped in 1% Eosin solution (Electron Microscopy Sciences, Hatfield, PA, 62534-15) to stain the ECM, then dehydrated prior to being mounted with xylene-based mounting media and covered with a coverslip in xylene-based mounting media. For cresyl violet staining, sections were dehydrated in a series of ethanol washes from 70% to 100% ethanol, then rehydrated in the opposite order followed by a rinse in distilled water prior to incubation in 0.25% cresyl violet in acetate buffer for 2-7 minutes. Sections were then dehydrated again in ethanol and differentiated in 0.25% glacial acetic acid in ethanol followed by xylene rinses prior to coverslip placement using a xylene-based mounting media. H&E and cresyl violet staining were used to assess lesion extent.

Cells in the cavity area were identified using the antibodies in **Table 4.2** and examined to determine in vivo differentiation. Lesion extent was assessed by examination

of lesion volume comparison between ipsilesional and contralesional side of each section. Lesion extent was assessed by examining shrinkage of ipsilesional side compared to contralesional side.

Statistics

A two way ANOVA with Bonferroni correction was performed between groups to determine significant differences in lesion extent. Spearman's rank correlation test was performed to correlate tissue loss to behavioral outcomes of tests that indicated significant enhancement of recovery.

Table 4.2: Antibodies used for in vitro and in vivo experiments.

Antibody Name	Antibody Specificity	Identification	In vitro/in vivo
O4	-	All stages of oligodendrocyte development	y/n
NG2	CSPG receptor	All stages of oligodendrocyte development	y/y
GFAP	Glial fibrillary acidic protein	Reactive astrocytes	y/y
b-III tubulin	Neuron- specific tubulin	Neurons	y/y
UTY	Y chromozome	Transplanted cells	n/y
bFGF receptor	Basic fibroblast growth factor	Transplanted cells	n/y
Laminin	Laminin I	Extracellular matrix in hydrogels	n/y
Collagen	Collagen I	Extracellular matrix in hydrogels	n/y

Behavioral assessment results

Postural instability test (PIT)

This test reveals the animals' ability to step in response to a shift in their center of gravity. Powerful data regarding compensation can also be acquired with this technique by testing the contralesional limb. The deficit was detected with this method, animals with injury on average had an increase in step length from ~11.4 cm / two steps to ~18 cm/ two steps with the ipsilesional limb (**Fig. 4.3a**). This increase indicates that the functional deficit caused by the contusion injury affects the reflexive behavior to stabilize with a shift in center of gravity over the forelimbs. Similarly, a compensatory behavior was observed in the contralesional limb, with contused animals stepping on average ~8.9 cm/ two steps compared to animals that received a laminectomy stepping ~11.4 cm/two steps (**Fig. 4.3b**). Unfortunately, no difference in functional recovery was observed between groups, nor was there an increase in functional ability throughout the experiment for any experimental group.

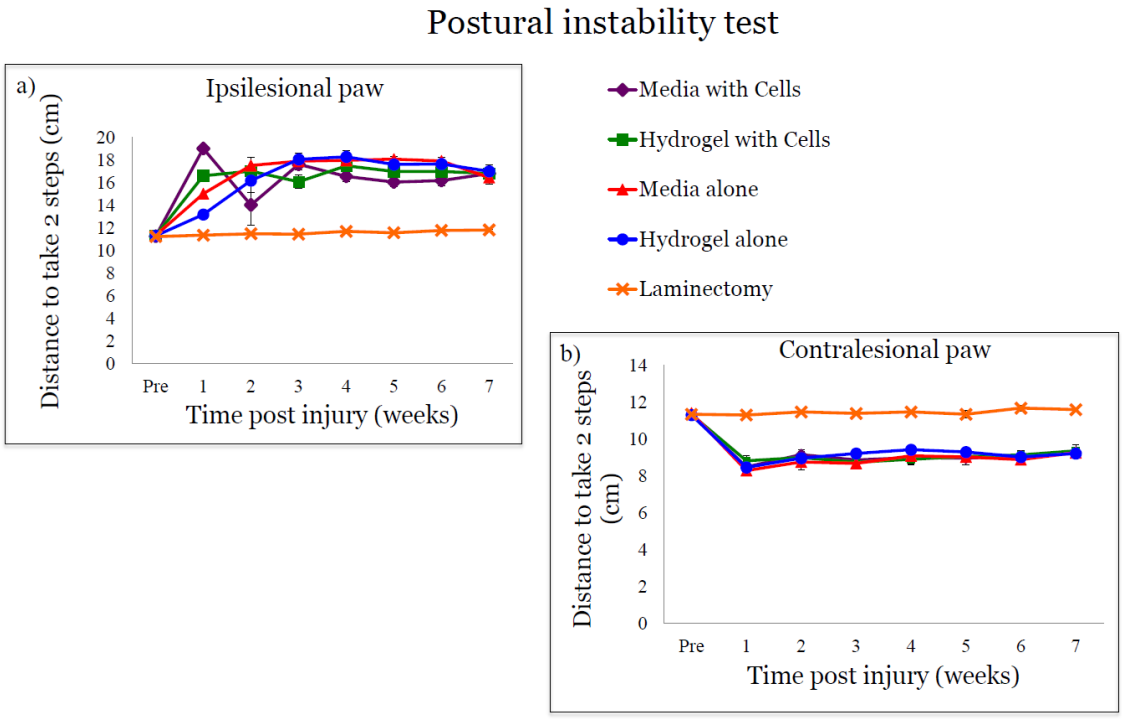

Figure 4.3: Postural instability test.
This test assessed the animals' reaction to a shift in center of gravity. (a) All groups presented a deficit in the function of their ipsilesional forelimb, however, there was no significant recovery in any group. (b) Assessment of compensation is also important when examining effects after a unilateral lesion. All groups expressed compensation on the contralesional limb, however, no change was observed throughout the 7 weeks of testing.

Forelimb step-alternation test

Assessing animals' ability to alternate stepping provides insight to the normal function of the animal under a forced motion task. A deficit was observed after injury and slight increase in functional ability in every group was observed greater than 60% of animals in all groups alternating steps by week 4 (**Fig. 4.4**), however, there was little difference observed between groups. The groups that received media with cells and the hydrogel alone had 75% of animals alternating (**Fig. 4.4**), however, this increase represents one animal difference in the groups and is therefore not considered significant.

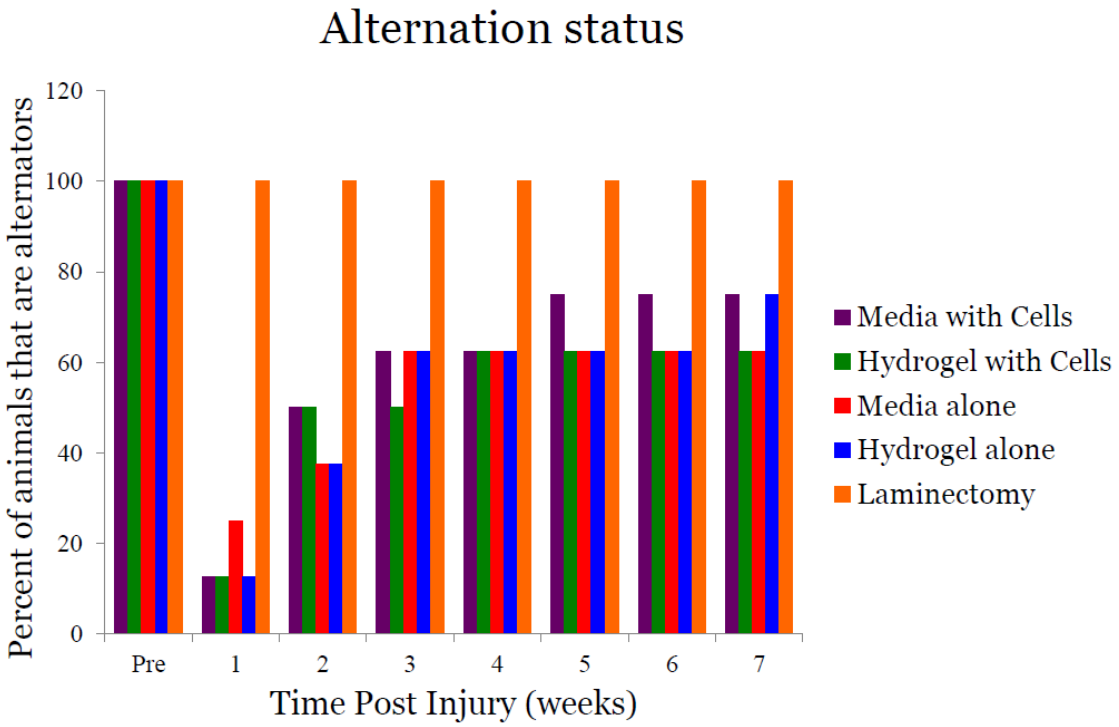

Figure 4.4: Forelimb step-alternation.
This test examined the functional outcomes after unilateral spinal cord injury with respect to normal stepping during locomotion. No significant difference were seen between groups, however there was improvement in all groups over the first three weeks after injury.

Vibrissae-elicited forelimb placing test

Animals were examined for their ability to respond to a sensory input from their whiskers to stabilize their bodies on the tabletop. A deficit was observed one week after injury, and an increase in function was observed in the two groups with hydrogel transplants. By week 5, the group with a hydrogel + cells transplant performed significantly better than the media alone and media with cells groups (**Fig. 4.5**). This increase represents some animals gaining the ability to reach for the table, though not able to support themselves on the table. Gaining the functional ability to reach from a resting position represents a significant increase in quality of life by providing some independence.

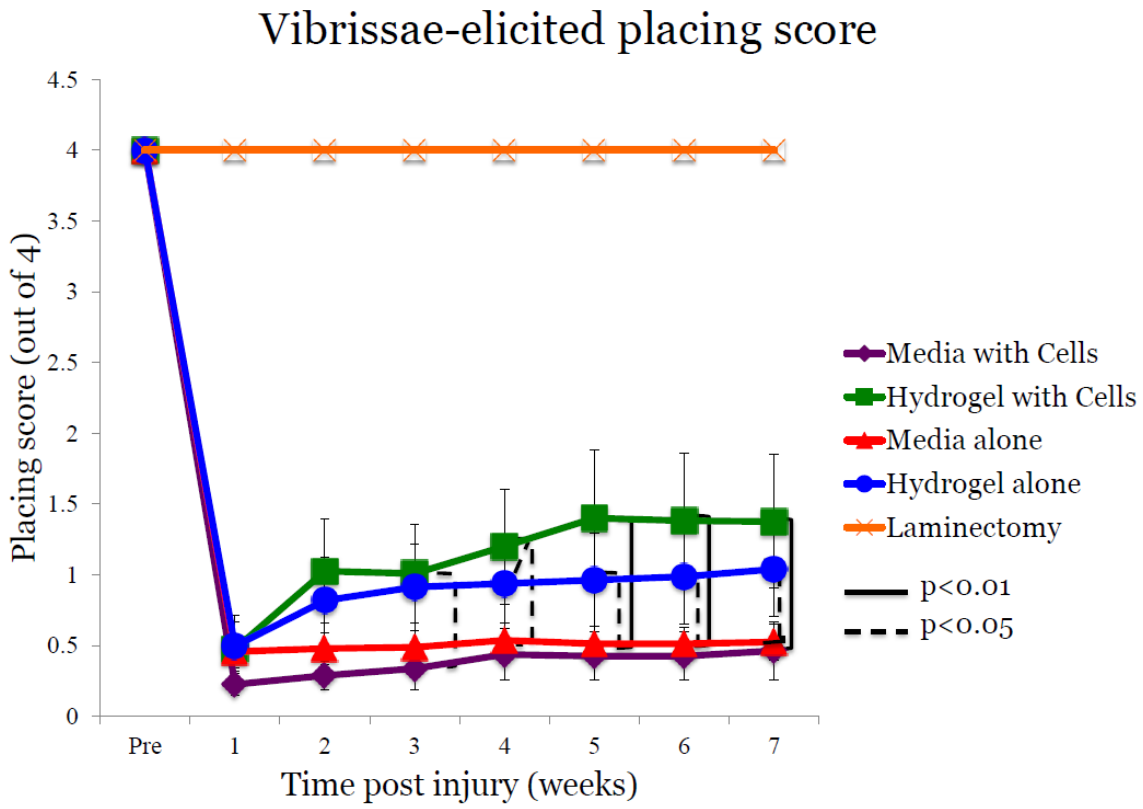

Figure 4.5: Vibrissae-elicited placing.
This test examined the animals sensory-motor function unrelated to reflex by examining a motor task in response to a sensory stimulus unrelated to the direct spinal reflex path. Significant improvement was seen two weeks after treatment in groups receiving hydrogel transplantations compared to media groups. Four weeks after treatment, the hydrogel + cells group improved above hydrogel alone group, however, statistical difference was not found between the two groups. The improvement represents animals having complete paralysis of the ipsilesional limb and improving to having the ability to move their limb forward to touch the table.

Cylinder paw use preference test

Animals were tested in free motion while exploring the walls of a vertical cylinder. This provides insight to relaxed, unprovoked behavior. A deficit was observed one week following injury and animals slowly regained some function over the following six weeks. Animals with a hydrogel + cells transplant performed significantly better by the sixth week after treatment than animals with a media alone transplant (**Fig. 4.6**). This implies that these animals are more likely to use their ipsilesional limb during normal motion. This follows from the placing data and supports the implication of increased independence with such recovery.

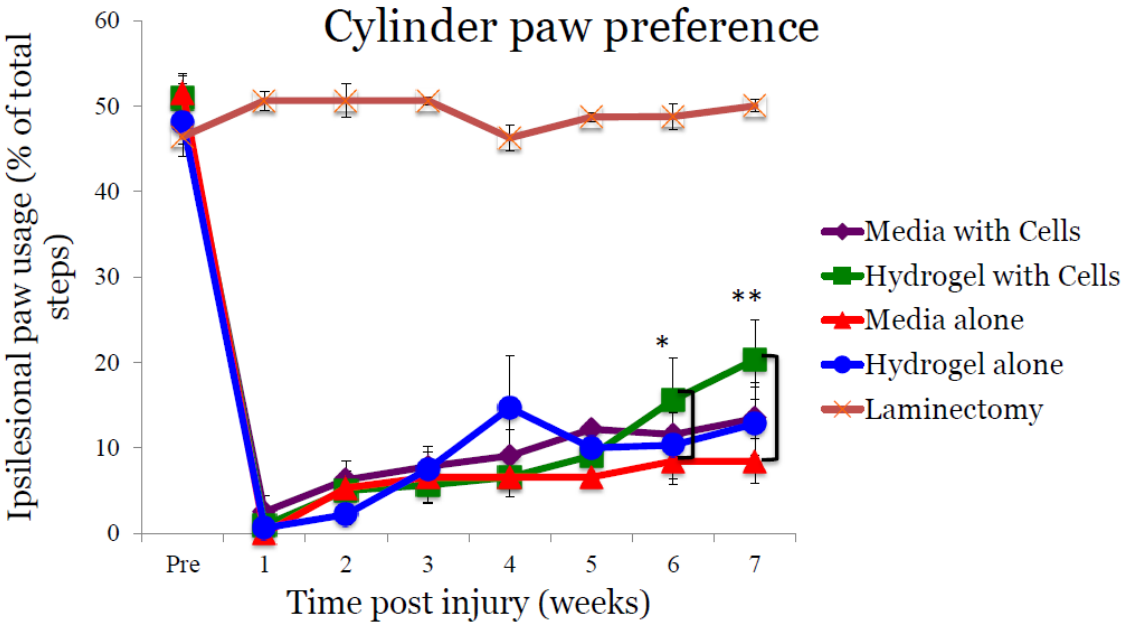

Figure 4.6: Cylinder paw preference.
Assessment of forelimb function with the cylinder paw preference test reveals an animal's ability to use their paws in a normal motion task. All groups exhibited a deficit immediately after surgery and showed improvement over the weeks following treatment. However, the hydrogel + cells group improved significantly over the media alone group by six weeks after injury. Note: * $p<0.05$, ** $p<0.01$

Pasta handling and eating

Rats were observed eating pasta, providing insight to paw use in a motivated, though not forced situation. A deficit was observed one week post injury, with most animals exclusively using their contralesional limb to eat the pasta. All groups showed some improvement in paw usage following injury, however, this increase was not significant (**Fig. 4.7**).

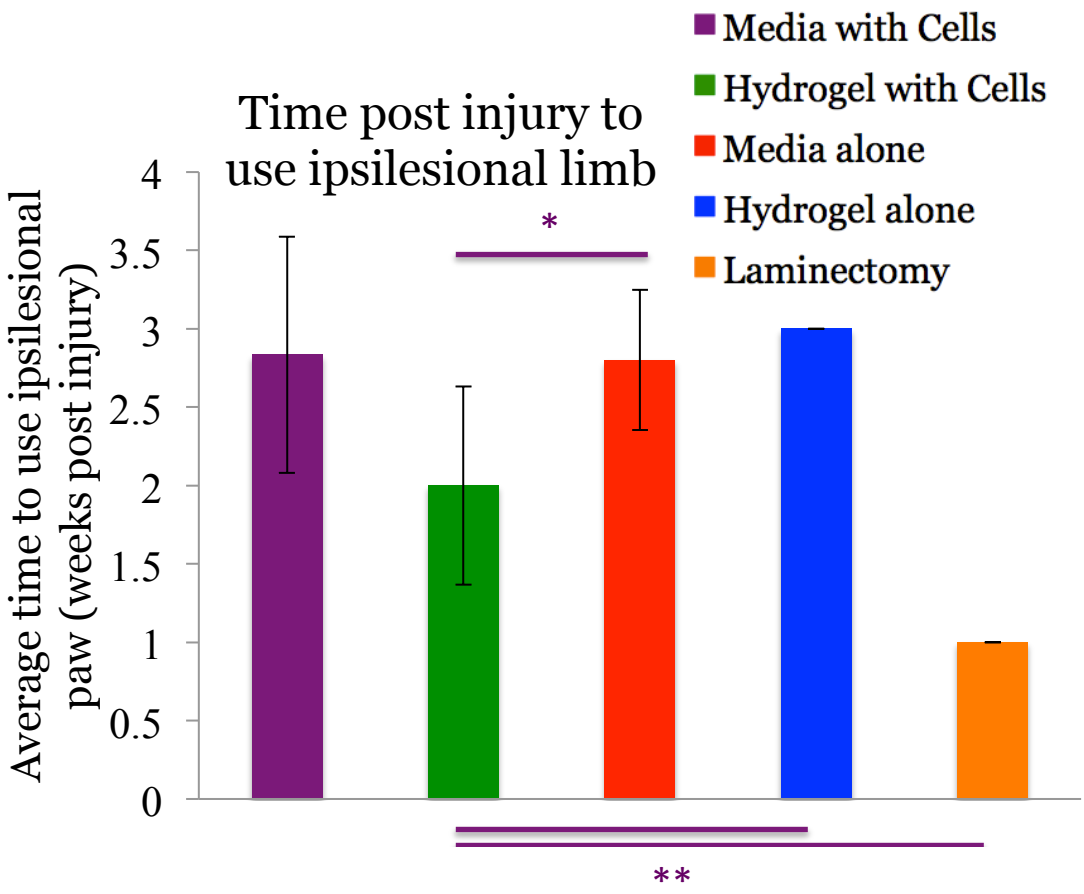

Figure 4.7: Pasta handling and eating test.
Assessment of time to use the ipsilesional limb after injury reveals the speed of recovery for each experimental group. 75% of animals in all groups recovered function by week 7, however, the average time to recover function was different between groups, with the hydrogel with cells group increasing the rate of recovery significantly. Note: *$p<0.05$., **$p<0.01$.

Grooming test

Another motivated sensory motor test was used to assess the animals' range of motion of their forelimbs. The grooming test provides motivation to the animals to use the full range of motion of their limbs. A deficit was observed one week post injury. Small differences between groups were observed, but no significant improvements were observed in any group (**Fig. 4.8**).

Analysis of behavioral assessment results

Few of the behavioral tests used exhibited significant differences between groups. These tests may not have been sensitive enough to detect the changes in functional recovery, and minimal functional recovery may have occurred over the timeframe of the experiment. This experiment was designed to examine recovery of function in response to remyelination and tissue sparing with treatment, however, a longer term experiment would reveal more extensive regeneration.

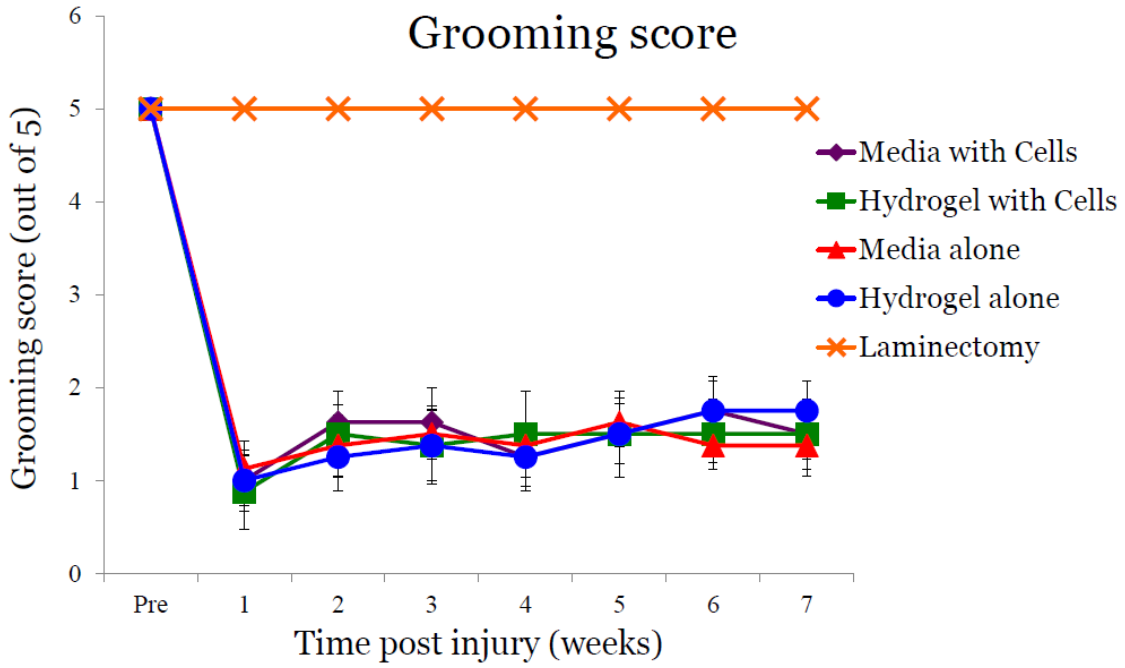

Figure 4.8: Grooming test.
Forelimb assessment using the grooming test allows researchers to examine the animals' response to a minimally stressful stimulus. Significant deficit was observed one week after injury, and all groups improved slightly over the following weeks. No differences were seen between groups.

Histological assessment results

Lesion extent assessment.

Lesion extent was assessed at two and six weeks post treatment. Significant differences in tissue loss based on lesion extent were observed between the hydrogel + cell transplantation group and the media alone transplantation group (**Fig. 4.9**) with the hydrogel + cell group losing 30.5% tissue whereas the media alone group tissue volume decreased by 51.7%. This may have been caused by the increased stability of the cavity or to the cellular response to the hydrogel transplants. Additionally, transplant groups with hydrogels were qualitatively assessed to have less collapse of the lesion area than media groups.

Hydrogel identification

The hydrogel was identified in the lesion area using collagen I and laminin I staining. The hydrogel appears to have integrated with the tissue well, with cells observed at the interface and in the lesion center in the hydrogel alone group as well as the hydrogel + cells group. This result implies interaction between the host tissue and the transplanted hydrogel. Additionally, little presence of the hydrogel 6 weeks after transplantation implies reorganization and absorption of the hydrogel protein components.

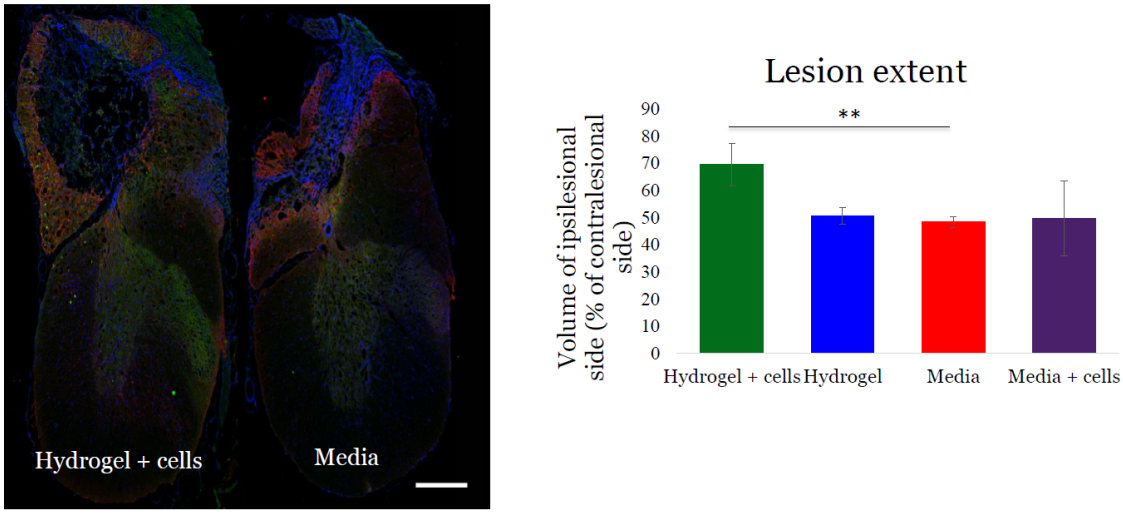

Figure 4.9: Lesion extent.
Lesion extent was calculated by examining the difference in tissue volume between the ipsilesional and contralesional sides of the spinal cord at the epicenter of the lesion. Significantly more tissue was present at the epicenter in the hydrogel + cell transplantation group ($p<0.01$) compared to media alone groups. Spinal cord sections were stained with GFAP, β-III tubulin, and DAPI. Note, scale bar is 500 μm, **p=0.01.

Scar formation and tissue response

Staining was performed to observe scar formation in the lesion area. Qualitative assessment of scarring revealed a more significant scar response in groups without hydrogel transplant, following the hypothesis that hyaluronic acid would interact with reactive astrocytes by providing signals to prevent further scarring. Examination of the morphology of the astrocytes at the lesion edge reveals a lower cellular density and a less organized scar in the hydrogel transplant groups (**Fig. 4.10**).

Differentiation

Transplanted cells were identified in the lesion using UTY and bFGF receptor staining. Additionally, differentiation of these cells and other cells in the lesion area was observed qualitatively. Some oligodendrocytes were present in the lesion cavity area of the hydrogel + cells group, and astrocytes were seen to have migrated throughout the lesion area in all groups, likely in response to infiltrating exogenous cells. In all groups, invading exogenous cells were present, implying a breach in the dura and making it impossible to assess differentiation of transplanted cells.

Analysis of histological results

Histological assessment reveals a successful transplantation of hydrogels and cells and provides insight to the behavior of the transplanted cells. Lesion volume reduction is a promising outcome to this therapy, as tissue sparing provides hope that regeneration may be successful.

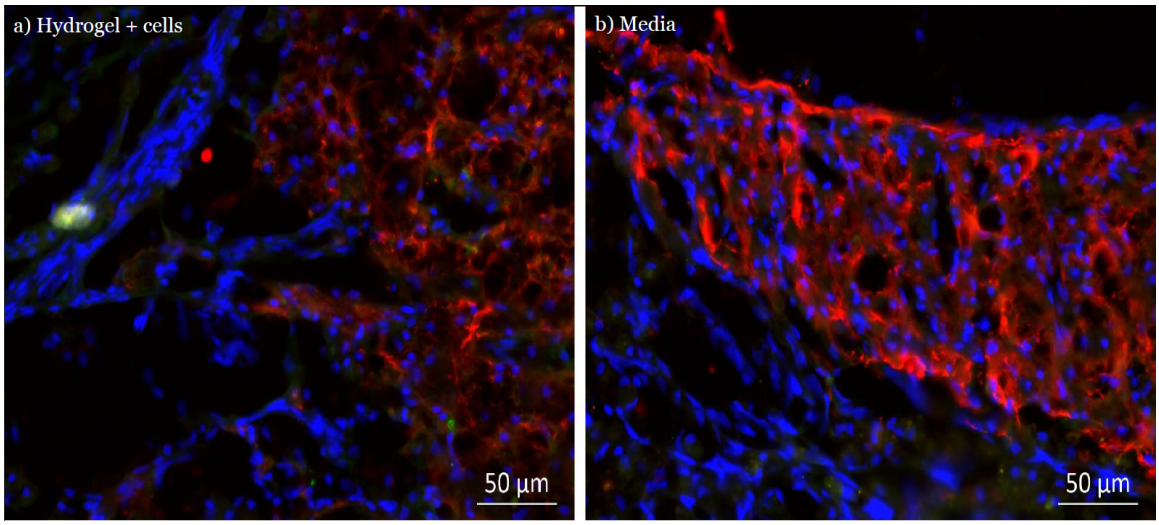

Figure 4.10: Scar formation.
Scar formation was examined to determine the response of reactive astrocytes to hydrogel transplantation. A different morphology of the scar as well as decreased GFAP staining was seen in hydrogel treated groups (a) compared to media groups (b). Qualitative assessment determined that the astrocytes making up the scar were interacting with hydrogels in the lesion.

Specific Aim 3 Summary

Transplantation of hydrogels optimized to direct neural progenitor cell differentiation toward oligodendrocytes was shown to be a promising treatment after spinal cord injury. Over a short-term experiment, tissue sparing was observed. The regenerative capacity of these therapies will need to be determined with longer-term experimentation. The histological outcomes with hydrogel treatment are promising in their ability to spare tissue. A cell tracking technique that has been verified needs to be implemented to examine differentiation of transplanted cells in vivo.

Chapter 5: Discussion and Conclusion

RESTATEMENT OF PROBLEM

Spinal cord injury is a debilitating injury that has insufficient therapeutic options to provide patients with functional recovery. Researchers are examining cell therapies to replace and enhance endogenous cell populations. When neural progenitor cells are transplanted, experiments are plagued by low cell survival and undirected differentiation. To attenuate the latter problem, biomaterials have been added to the transplantation protocol. However, many of these biomaterials do not play a specific role beyond supporting cell viability. The cells receive cues from the biomaterials used, but without control of the material's mechanical and chemical properties of these materials, many researchers report undesirable differentiation of the transplanted cells. Research has recently shifted to modulation of the materials used for transplantation to direct this differentiation by conjugation with growth factors or the use of microparticles for sustained drug delivery. This dissertation presents a novel and exciting application of natural components to direct the differentiation of neural progenitor cells toward oligodendrocytes using cues present during development. Though the effects of mechanical properties on neural progenitor cell differentiation has been examined in culture [134], this technique was previously not applied to control in vivo differentiation of cells transplanted after spinal cord injury.

By controlling the mechanical properties of this hydrogel, cell differentiation can be directed toward oligodendrocytes, an important cell type to enhance axonal growth. Additionally, the composition of the hydrogel (collagen, hyaluronic acid, and laminin),

provides signals to the cells to induce migration and differentiation toward this cell type. Differentiation of spinal neural progenitor cells was examined in vitro and in vivo. Additionally, this study examined the effects of such a transplant on spinal cord tissue sparing after injury.

6.1. Conclusion

For this project, the intrinsic bioactivity of natural biomaterials was harnessed to direct cellular differentiation in vitro and in vivo in pursuit of the goal to encourage recovery after central nervous system trauma. However, the same material and concepts can be applied to regenerative engineering in many soft tissues. This biomaterial provides two key properties of a successful biomaterial (1) tunable mechanical properties and (2) native extracellular matrix cues. Additionally, the composition of this hydrogel is exclusively natural components, which allows cells to reorganize and rebuild the structure.

SPECIFIC AIM 1: OPTIMIZE A NOVEL INJECTABLE HYDROGEL COMPRISED OF EXTRACELLULAR MATRIX COMPONENTS TO MIMIC MECHANICAL AND BIOCHEMICAL PROPERTIES OF NATIVE SPINAL CORD TISSUE.

The research presented here aimed to design an engineered matrix for soft tissues using natural components, such as collagen, hyaluronic acid, and laminin. In Specific Aim 1, multiple hydrogel compositions were developed. These hydrogels were extensively characterized with respect to the mechanical properties, gelation kinetics, and hyaluronic acid diffusion. The mechanical properties were soft enough to present similar cues to cells as neonatal spinal cord tissue. The addition of each component affected the mechanical properties of the collagen base material. Independent control of mechanical

properties and extracellular matrix components may be important in biomaterial design to provide specific cues for each application. However, the mechanical properties here mimic the neonatal nervous tissue of interest sufficiently.

Future

More precise control of mechanical and extracellular matrix components of this biomaterial will make it translatable to multiple applications. Development of an injectable, thermally gelling material for transplantation with controlled mechanical properties not linked to composition will provide a material that will more easily assist researchers in distinguishing important characteristics for biomedical applications.

SPECIFIC AIM 2: EXAMINE THE DIFFERENTIATION OF NEURAL PROGENITOR CELLS TOWARD OLIGODENDROCYTES EMBEDDED IN DIFFERENT HYDROGEL COMPOSITIONS.

Cellular differentiation in the multi-component natural-based hydrogels used in this project was robust with the cell types examined making up nearly 100% of all cells. Three cell types were examined, astrocytes (identified with GFAP), neurons (identified with β-III tubulin), and oligodendrocytes (identified with O4 and NG2). All cell types were observed in all four hydrogel types, however, the percentage of oligodendrocytes present in the three-component hydrogels was greater than in the other hydrogel types. This differentiation was likely in response to the cues provided by the hydrogels.

Recently, the response of progenitor cells to mechanical stiffness has become an exciting field for biomaterials engineers and stem cell scientists. In 2006, a seminal work out of the Discher group introduced the response of mesenchymal stem cells to differing mechanical properties and many researchers have continued to examine the responses of

progenitor and stem cells to mechanical cues [128]. Many researcher, however, examine cellular response to mechanical cues in two dimensional culture conditions. Three dimensional cell culture provides a more representative examination of cellular response in vivo and therefore provides a more physiologically relevant prediction of cell behavior after transplantation.

However, mechanical properties of these hydrogels were modulated based on the composition of the hydrogel. This limited the ability to distinguish the importance of mechanical properties compared to the extracellular matrix component signals on cell behavior. It is important to distinguish the relevance of each signal type to the differentiation of neural progenitor cells.

Future

Determining the cues that are most important for directing progenitor cell differentiation will provide researchers with better tools to control the environment of transplanted cells to encourage regeneration in the central nervous system. To achieve these goals, photocrosslinkable hyaluronic acid hydrogels used previously in the Schmidt lab [134] or poly(ethylene glycol) hydrogels with tunable mechanical properties [158] could be used to provide specific mechanical cues to cells in culture. With controlled mechanical properties, ECM components can be introduced to the hydrogels to provide specific cues to the differentiating cells. This approach would provide the means to determine the effects of each component whereas combinatorial effects of multiple chemical components could be studied without altering the mechanical properties significantly.

SPECIFIC AIM 3: IMPLEMENT AN INJECTABLE HYDROGEL-CELL SYSTEM INTO AN IN VIVO MODEL TO ASSESS FUNCTIONAL AND ANATOMICAL RECOVERY AFTER SCI.

A unilateral cervical contusion model of spinal cord injury in the rat provided an optimal transplantation site and a large selection of behavioral assays to assess the deficit and recovery. This model represents a clinically relevant population and allows for examination of compensation and deficit.

The six behavioral tests selected examined different aspects of functional recovery. Many of them, however, were not sufficiently sensitive to reveal a difference between groups. Three of the six tests showed improvement after treatment with the optimized hydrogel and cells over the control by six weeks after transplantation.

Histological assessment two and six weeks after treatment provided insight to the cellular behavior in vivo. Results followed the hypothesis that tissue sparing would increase with hydrogel transplantation. Cell differentiation appears to follow the same trend as in vitro, supporting the hypothesis that the hydrogel signals would affect the cellular behavior in vivo and in vitro. The transplanted spinal progenitor cells used did not provide a sufficient means of tracking their location. Therefore, this experiment will need to be repeated with more identifiable cells.

Future

This experiment assessed early timepoint tissue sparing and lesion effects after transplantation of neural progenitor cells and hydrogels. However, regeneration is expected to start 8 weeks post transplantation. A more long-term experiment would reveal the effects of this therapy on functional regeneration. Additionally, the combination of a well-controlled hydrogel environment and finely tuned growth factor

delivery; for example the delivery of platelet derived growth factor or ciliary neurotrophic factor [111] may provide a more optimal environment for oligodendrocyte maturation in vivo.

CONCLUSION

In conclusion, this research demonstrated a novel application of a biomaterial/progenitor cell construct to provide a possible path for developing future treatments of spinal cord injuries. Future work should be directed toward refinement of this or similar materials for more control of the tunability of each property. With this additional control, this natural biomaterial could be applied to a multitude of regenerative applications.

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